PATENT APPLICATION

#15
11/6/02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

Robert G. Lamb

Group Art Unit: 1615

Application No.: 09/670,346

Examiner: G. Kishore

Filed: September 27, 2000

Attorney Dkt. No.: 021941-00001

For: VITAMIN E PHOSPHATE/PHOSPHATIDYLCHOLINE LIPOSOMES TO PROTECT FROM OR AMELIORATE CELL DAMAGE

FIRST DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Dr. Robert G. Lamb, hereby declare and state:

I am the sole inventor for the above-identified patent application.

My expertise is in the field of lipid metabolism. My curriculum vitae is attached to this declaration as Attachment A.

My invention is directed to Vitamin E phosphate (VEP) encapsulated in liposomes formed of a particular phosphatidylcholine. Specifically, surprising results have been obtained when the material added to VEP and processed as shown in Example 8 of the patent application to form a liposome (the liposome former) is polyenylphosphatidylcholine (PPC).

I have performed or overseen the performance of a number of comparative experiments studying the inventive Vitamin E phosphate/polyenylphosphatidylcholine (VEP/PPC) agent in biological assays designed to examine the effects of the agent on

protecting against aging and injury of cells challenged with ethanol. A summary of my findings is set forth as follows.

I tested the inventive VEP/PPC agent against VEP encapsulated in liposomes formed of chicken egg phosphatidylcholine (VEP/EPC). Both agents were prepared identically to the procedures outlined in the patent application, with the exception that the two different phosphatidylcholine liposome formers were used.

The data in the Table below demonstrates that VEP/PPC is almost five times more effective in reducing the adverse effects of ethanol on cultured liver cells than the comparative agent VEP/EPC. More specifically, my tests revealed that when cultured liver cells are exposed to ethanol, the treatment of the liver cells with the inventive VEP/PPC causes the reduction of cell function from the exposure to ethanol to be almost five times less than the reduction in cell function that occurs with the treatment of such liver cells with the comparative agent VEP/EPC. In fact, in the experiments, the inventive VEP/PPC essentially blocked the adverse cellular effects of ethanol.

The experimental procedure was as follows.

Cultured liver cells were incubated for 24 hours with 100 mM ethanol in the presence of (1) water, (2) VEP/EPC, or (3) VEP/PPC. Agent-dependent alterations in cell function were determined by measuring phosphatidylcholine biosynthesis. Similar results were obtained in three separate preparations of cultured cells, all of which are reflected in the Table set forth below.

Control cells are expressed as 100%, meaning full cellular function. A reduction below 100% represents a decrease in cell function. Cells exposed to 100 mM ethanol for 24 hours exhibited a significant ($p < 0.01$) reduction in cell function, down 63% from control cells.

Cells incubated with 100 mM ethanol for 24 hours in the presence of VEP/EPC (15 μM VEP and 30 μM EPC) showed a significant ($p < 0.01$) reduction in the adverse cellular effects of ethanol, as cellular function was only decreased by 33%.

Surprisingly, however, cells incubated with 100 mM ethanol for 24 hours in the presence of VEP/PPC (15 μM VEP and 30 μM PPC) only displayed a 7% reduction in cell function. This demonstrates that VEP/PPC was significantly ($p < 0.01$) better in reducing the adverse cellular effects of ethanol than VEP/EPC. In fact, VEP/PPC was found to be almost five times more effective than VEP/EPC in reducing the adverse effects of ethanol on cells.

The results are displayed in the following Table:

% of PC biosynthesis in cells incubated with the listed Addition, as compared to Control

Additions	**% Control ± SEM**
None	100 ± 2
Ethanol	37 ± 1 *
Ethanol + VEP/EPC	67 ± 4 **
Ethanol + VEP/PPC	93 ± 3 ***

* Level of significance from control (none) is $p < 0.01$
** Level of significance from Ethanol is $p < 0.01$
*** Level of significance from Ethanol + VEP/EPC is $p < 0.01$

These results demonstrate that the VEP/PPC of the patent application is inventive due to its completely surprising superiority at protecting cells from injury.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of

Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: 10/22/02

Robert Lamb

Dr. Robert G. Lamb

CURRICULUM VITAE

I. CREDENTIALS AND EXPERIENCE

Name: Robert G. Lamb

Born: August 20, 1944
Portland, Oregon

Marital Status: Married to Debby Ann Hougland
Three children:
Robert Gregory
Christopher Scott
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EDUCATION:

B.A. in chemistry, University of North Carolina at Chapel Hill,1967.

Ph.D. in biochemistry, minor in chemistry and zoology, University of North Carolina at Chapel Hill, 1970. Dissertation Title: Acylation of glycerol-3-P by rat liver microsomes.

Postdoctoral Fellow, Division of Clinical Pharmacology, Department of Medicine, University of North Carolina, 1970-72. Research Project: Mechanisms of action of lipid-lowering agents such as clofibrate.

POSITIONS

Lecturer, Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina, 1971-74.

Instructor, Department of Medicine and Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina July 1972 - June 1973.

Assistant Professor, Department of Pharmacology and Medicine, University of North Carolina School of Medicine, Chapel Hill, North Carolina, July 1973 - June 1974.

Assistant Professor, Department of Pharmacology and Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, July 1974 - June 1980.

Associate Professor, Department of Pharmacology and Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, July 1980 - June 1990.

Professor, Department of Pharmacology and Medicine, Medical College of Virginia, Virginia Commonwealth University. Richmond, Virginia, July 1990 - present.

II. TEACHING

I consider teaching as one of my most important functions as a VCU faculty member. My teaching load is "heavy" (100-110 hours/year) since I teach medical, dental, pharmacy, graduate and undergraduate (honors) students. I have retained student evaluations (>2000) of my teaching performance since 1981. Over 90% of the students rated my teaching as very good to excellent. I have incorporated a computer simulation (Pharmkin) of the blood drug concentrations in patients into my pharmacokinetic lectures. I have been responsible for developing the use of "Pharmkin" in the computer center (Tompcat). The student's response to this learning technique is "excellent".

COURSES

A. PHARMACOLOGY 536, General Pharmacology, a comprehensive course for graduate students. I teach and coordinate the pharmacokinetic section of this course **(22 contact hours)**. Students develop a fundamental understanding of the absorption, distribution, metabolism,excretion and toxicology of drugs and are required to solve pharmacokinetic problems using the "Pharmkin" simulation of plasma drug concentrations in patients.

B. PHARMACOLOGY 609, General Pharmacology and Pain Control, basic pharmacology principles for dental students. I give lectures on mechanisms of drug action, pharmacokinetics, drug interactions and lipid-lowering agents **(16 contact hours)**. Students are required to solve pharmacokinetic problems using the "Pharmkin" simulation of plasma drug concentrations in patients.

C. MII PHARMACOLOGY, General Pharmacology for second year medical students. **I have been the COURSEMASTER of the Medical School pharmacology course.** I lecture on mechanisms of drug action, pharmacokinetics and drug interactions **(21 contact hours)**. Students are required to solve pharmacokinetic problems using the "Pharmkin" simulation of plasma drug concentrations in patients.

D. PHARMACOLOGY 603 AND 604 General Pharmacology for Pharmacy Students**. I am the COURSEMASTER of the Pharmacy Pharmacology Course.** I lecture on mechanisms of drug action, pharmacokinetics, factors influencing drug action and drug interactions **(20 contact hours).** Students are required to solve pharmacokinetic problems using the "Pharmkin" simulation of plasma drug concentrations in patients (required laboratory).

E. PHARMACOLOGY 537, General Pharmacology for Graduate Students. I lecture on lipid and lipoprotein metabolism and mechanisms of action of lipid-lowering agents **(2 contact hours).**

F. PHARMACOLOGY 535, Principles of Toxicology for Graduate Students. I lecture on hepatotoxicology **(2 contact hours).**

G. PHARMACOLOGY 638, Advanced Toxicology. I lecture on mechanisms of chemical dependent cell injury **(2 contact hours).**

H. PHARMACOLOGY ELECTIVE FOR M IV STUDENTS. I lecture on lipoprotein metabolism and mechanism of action of lipid-lowering agents **(3 contact hours). I have benn the COURSEMASTER of this course.**

I. PHARMACOLOGY 597, Introduction to Pharmacological Research. **I am the COURSEMASTER of this course**. I also lecture on the research conducted in my laboratory **(4 contact hours)**. Students that select my laboratory for a rotation then spend approximately 12 hours per week for 6-8 weeks doing supervised research.

J. PHARMACOLOGY 697, Directed Research in the laboratory. **Six students have received their Ph.D. under my supervision.**

K. Introduction to Clinical Pharmacokinetics. An "Honors Module" for junior and senior undergraduates interested in medicine, dentistry, pharmacy and nursing (15 contact hours). I lecture on mechanisms of drug action, pharmacokinetics and drug interactions. I am the coursemaster of this course and the course meets in both the Fall and Spring semesters **(30 contact hours for the year).**

STUDENTS, POSTDOCTORAL FELLOWS, VISITING SCIENTISTS AND ASSOCIATES

A. Craig K. Wood, Ph.D.

1974-1978, Ph.D.-1978, Dissertation Title: The effect of ethanol on hepatic glyerolipid biosynthesis.

B. Dorie W. Schwertz, Ph.D.

1977-1981, Ph.D.-1981, Dissertation Title: The effect of carbon tetrachloride on rat hepatocellular membrane glycerolipid synthesis, degradation and content.

C. Judith Ann Woods, Ph.D.

1977-1981, Ph.D.-1981, Dissertation Title: The acute effects of streptozotocin-induced diabetes on rat hepatic glycerolipid biosynthesis.

D. John B. Coleman, Ph.D.

1983-1987, Ph.D.-1987, Dissertation Title: The role of biotransformation in the activation of phospholipase C by carbon tetrachloride and related hydrocarbons.

E. Jack W. Snyder, M.D., Ph.D.

1983-1987, Ph.D.-1987, Dissertation Title: The role of phospholipid metabolism in ethanol- and acetaldehyde-initiated hepatocyte injury.

F. Bernard F. Schneider, Ph.D.

1979, Postdoctoral Fellow in Gastroenterology. Bernie spent one year in the laboratory learning electron microscopy techniques (with Dr. Francine Cabral) and conducting some preliminary studies on the effects of radiation on liver cell function and structure.

G. Johana E. Groener, Ph.D.

Johana was a visiting scientist for one year (1979). She learned how to prepare primary cultures of adult rat hepatocytes and worked with Dr. Tom Knauer on the effects of STZ-dependent diabetes on hepatic lipase activity.

H. Thomas E. Knauer, Ph.D.

Tom was a research associate (1979-1980) and we collaborated on studies using streptozotocin to alter hepatic glycerolipid biosynthesis and hepatic lipase activity.

I. Steven Wyrick, Ph.D.

Steve visited my laboratory (1977) to learn techniques that he used to complete his dissertation project with Dr. Piantadosi. This project was a result of a collaboration that started in Chapel Hill before I came to MCV.

J. Lynn Poindexter

A student apprentice of the MCV Health Careers Opportunity Program for Minority Students (1982).

K. Hiep Tran

A student apprentice of the MCV Health Careers Opportunity Program for Minority Students (1983).

L. Earlene Russell

A student apprentice of the MCV Health Careers Opportunity Program for Minority Students (1984).

M. Antoniette Rogers

A student apprentice of the MCV Health Careers Opportunity Program for minority Students (1987).

N. Sunile Mathews

A student apprentice of the MCV Health Careers Opportunity Program for Minority Students (1988).

O. Melvin Jarrett

A student apprentice of the MCV Pharmacology and Toxicology Department's Program for Minority Students (1989).

P. Melba Williams

A student apprentice of the MCV Health Careers Opportunity Program for Minority Students (1990).

Q. Ray McKenzie

A student apprentice of the MCV Pharmacology and Toxicology Department's Program for Minority Students (1990; 1991).

R. Tam McRae

A student apprentice of the MCV Health Careers Opportunity Program for Minority Students (1991).

S. John Koch, Ph.D.

John worked in the laboratory as a postdoctoral fellow (1989-91). He was attempting to resolve the molecular biology of several ambiquitous enzymes that play key roles in regulating hepatic glyerolipid metabolism.

T. Cheron Constance

A student apprentice of the MCV Health Careers Opportunity Program for Minority Students (1993).

U. Shiva Merat (Huband), Ph.D

1991-1996, Ph.D.-1996. Dissertation Title: Influence of ethanol, cocaine or both on cultured rat liver cell viability and phospholipid metabolism.

V. Chris Kirkup, M.S.

1993-1995, M.S.-1996. Thesis Title: The combined effects of acetaminophen and ethanol on cultured rat hepatocytes: metabolism-dependent generation of reactive oxygen species as a mechanism of injury.

W. Melinda Davila

A student apprentice of the MCV Pharmacology and Toxicology Department's Program for Minority Students (1997).

X. Kulsom Quarishi

A school teacher apprentice of the MCV Pharmacolgy and Toxicology Department's Program for Minority Students (1997).

III. SCHOLARSHIP

My research career has primarily focused on understanding how the liver regulates glycerolipid metabolism to meet the various demands of energy storage, lipoprotein biosynthesis, membrane phospholipid synthesis and hydrolysis and bile formation. In order to achieve these goals, we have developed a system of primary cultures of adult rat hepatocytes that mimics the liver cell's response to various agents in the intact animal. This in vitro model has been extensively used by this laboratory to determine:

1) the mechanisms by which agents such as ethanol, cocaine, acetaminophen, halogenated hydrocarbons, etc. produce cell injury;

2) that oxyradical-dependent cell injury and death may be due in part to alterations in the structure, hydrolysis and biosynthesis of membrane phospholipids such as PC;

3) how agents regulate key enzymes such as phosphatidate phosphohydrolase, phosphocholine cytidylyltransferase and phospholipiase C;
4) how lipid-lowering agents such as clofibrate and gemfibrozil reduce plasma lipid levels;
5) the hepatotoxic potential of agents alone and in combination;
6) the cytoprotective and antioxidant potential of vitamin E phosphate (VEP), phosphatidylcholine (PC) or combinations of VEP and PC.

PUBLICATIONS (CHAPTERS, ARTICLES AND PATENTS)

Fallon, H.J., and R.G. Lamb. Acylation of sn-glycerol-3-phosphate by cell fractions of rat liver. J. Lipid Res. 9:652, 1968.

Lamb, R.G., and H.J. Fallon. The formation of monoacylglycerophosphate from sn-glycerol-3-P by a rat liver particulate preparation. J. Biol. Chem. 245:3075, 1970.

Lamb, R.G. and H.J. Fallon. Inhibition of monoacylglycerophosphate formation by chlorophenoxyisobutyrate and betabenzalbutyrate. J. Biol. Chem. 247:1281-1287, 1972.

Fallon, H.J., L.L. Adams, and R.G. Lamb. A review of studies on the mode of action of clofibrate and betabenzabutyrate. Lipids, 7:106-109, 1972.

Lamb, R.G., P.M. Hill, and H.J. Fallon. Inhibition of palmitoyl CoA deacylase by chlorophenoxyisobutyrate and betabenzalbutyrate. J. Lipid Res. 14:459-465, 1973.

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Fallon, H.J., J. Barwick, R.G. Lamb, and H. Van den Bosch. Studies of rat liver microsomal diglyceride acyltransferase and choline phosphotransferase using microsomal bound substrate: The effects of high fructose intake. J. Lipid Res. 16:107-115, 1975.

Hall, I.H., R.G. Lamb, M.H. Mar, G.L. Carlson and C. Piantadosi. Cycloalkamones 5: synthesis, distribution and effects on triglyceride metabolism. J. Pharm. Sciences. 64:235, 1975.

Lamb, R.G. and H.J. Fallon. The effect of high sugar intake on the esterification of dihydroxyacetone phosphate by rat liver microsomes. J. Lipid Res. 17:406-411, 1976.

Fallon, H.J., J. Barwick, R.G. Lamb and H. Van den Bosch (1976). Studies on the specificity of the reactions of glycerolipid biosynthesis in rat liver using membrane-bound substrates. Lipids. R. Paoletti, G. Procellati, and G. Jacini editors. Raven Press, New York City. Volume 1 67-74.

Lamb, R.G., S.D. Wyrick and C. Piantadosi. Hypolipidemic activity of in vitro inhibitors of hepatic and intestinal sn-glycerol-3-phosphate acyltransferase and phosphatidate phosphohydrolase. Atherolsclerosis 27:147-154. 1977.

Fallon, H.J., R.G. Lamb and S. Jamdar. Phosphatidate phosphohydrolase and the regulation of glycerolipid biosynthesis. Biochem. Soc. Trans. 5: 37-40, 1977.

Lamb, R.G., C.K. Wood, B.M. Landa, P.S. Guzelian, and H.J. Fallon. Studies of the formation and release of glycerolipids by primary monolayer cultures of adult rat hepatocytes. Biochim. Biophys. Acta 489: 318-329, 1977.

DiMenna, W.S., C. Piantadosi, and R.G. Lamb. Synthesis of potential hypolipidemic agents. Reaction of substituted phenyl 2,3-epoxypropyl ethers with adenine, uracil and thymine. J. Med. Chem. 21: 1073-1076, 1978.

Wood, C.K. and R.G. Lamb. The effect of ethanol on glycerolipid biosynthesis by primary monolayer cultures of adult rat hepatocyte. Biochim. Biophys. Acta 572: 121-131, 1979.

Lamb, R.G., C.K. Wood, and H.J. Fallon. The effect of acute and chronic ethanol intake on hepatic glycerolipid biosynthesis in the hamster. J. Clin. Invest. 63: 14-20, 1979.

Guzelian, P.S., R.G. Lamb, and H.J. Fallon. Effects of hormones on changes in cytochrome P450, prolylhydroxylase and glycerol phosphate acyltransferase in primary monolayer cultures of parenchymal cells from adult rat liver. Yale J. Biol. and Med. 52: 5-12, 1979.

Lamb, R.G. and W.L. Banks, Jr. Effect of hydrazine exposure on hepatic triglyceride biosynthesis. Biochim. Biophys. Acta 574: 440-447, 1979.

Lamb, R.G., T.G. Gardner, and H.J. Fallon. Studies on the incorporation of sn-[1,3-^{14}C] glycerol-3-phosphate into glycerolipid by intestinal mucosa. Biochim. Biophys. Acta 619:385-395, 1980.

Lamb, R.G. and W.L. Dewey. Effect of morphine exposure on mouse liver triglyceride formation. J. Pharmacol. Exp. Ther. 216: 496-499, 1981.

Woods, J.A., Knauer, T.E. and Lamb, R.G. The acute effects of streptozotocin-induced diabetes on rat liver glycerolipid biosynthesis. Biochim. Biophys. Acta 666: 481-492, 1981.

Knauer, T.E., Woods, J.A., Lamb, R.G., and Fallon, H.J. Hepatic triacylglycerol lipase activities after induction of diabetes and administration of insulin or glucagon. J. Lipid Res. 23: 631-637, 1982.

Lamb, R.G. and Schwertz, D.W. The effects of bromobenzene and carbon tetrachloride exposure in vitro on the phospholipase C activity of rat liver cells. Toxicol. Appl. Pharmacol. 63: 216-229, 1982.

Wei, E.P., Lamb, R.G. and Kontos, H.A. Increased phospholipase C activity after experimental brain injury. J. Neurosurgery 56: 695-698, 1982.

Schwertz, D.W. and Lamb, R.G. The influence of carbon tetrachloride metabolism on the carbon tetrachloride-induced activation of rat liver cell phospholipase C activity. Toxicol. Appl. Pharmacol. 65: 402-412, 1982.

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Lamb, R.G., McCue, S.B. Taylor, D.R. and McGuffin, M.A. The role of phospholipid metabolism in bromobenzene- and carbon tetrachloride-dependent hepatocyte injury. Toxicol. Appl. Pharmacol. 75:510-520,1984.

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Lamb, R.G., K. Foster and M. McGuffin. A distinction in vitro between rat liver phosphatidate phosphatase and phospholipase C Biochim. Biophys. Acta, 921:67-74, 1987.

Lamb, R.G., J.W. Snyder and J.B. Coleman. New trends in the prevention of cell death: Modificators of calcium movement and of membrane phospholipid metabolism. In Liver Drugs: from experimental pharmacology to therapeutic application. Testa, B. and Perrisoud, D., Eds. CRC Press, Inc. Boca Raton, FL Chapter 5, 1988.

Coleman, J.B., Condie, L.W. and Lamb, R.G. The influence of CCl_4 biotransformation on the activation of rat liver Phospholipase C in vitro. Toxicol. Appl. Pharmacol. 95:200-207,1988.

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Lamb, R.G., Koch, J.C., Bush, S.R. and McElderry, J.S. An enzymatic explanation of the differential effects of oleate and gemfibrozil on cultured heaptocyte triacylglycerol and phosphatidylcholine biosynthesis and secretion. Biochim. Biophys. Acta 1165:299-305, 1993.

Nanji, A.A., Zhao, S., Lamb, R.G., Hossein Sadzadeh, S.M., Dannenberg, A.J. and Waxman D.J. Changes in microsomal phospholipases and arachidonic acid in experimental alcoholic liver injury: relationship to cytochrome P-450 2E1 induction and conjugated diene formation. Alcoholism: Clin. Exp. Res. 17:598-603, 1993.

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Nanji, A.A., Zhao, S., Lamb, R.G., Dannenberg, A.J., Sadrzadeh Hossein, S.M. and Waxman, D.J. Changes in Cytochromes P-450, 2E1, 2B1, and 4A, and phospholipases A and C in the intragastric feeding rat model for alcoholic liver disease: relationship to dietary fats and pathologic liver injury. Alcoholism: Clin. and Exp. Res. 18:902-906, 1994.

Lamb, R.G., Harper, C.C., Rzigalinski, B.A. McKinney, J.S. and Ellis, E.F. Alterations in phosphatidylcholine metabolism of stretch-injured cultured astrocytes J Neurochem. 68:1904-1910, 1997.

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Lamb,R.G. Vitamin E Phosphate/Phosphatidylcholine Liposomes to Protect from or Ameloiorate Cell Damage (US patent application), October, 2000.

Lamb, R.G. Test for Oxidative Stress Using Cell Suspensions. U.S. Patent # US 6,218,130B1, 4-17-2001.

Huband, S.M., Harris, L.S., Humphrey, S.B. and Lamb, R.G. Influence of cocaine, alcohol or both on cultured rat liver cell viability and phospholipid metabolism. (in preparation for submission)

Huband, S.M. and Lamb, R.G. Heptocellular phospholipases A and C are activated by acetaldehyde-dependent free radicals.(in preparation for submission)

Kirkup, C.T., Humphrey, S.B. and Lamb, R.G. The combined effects of acetaminophen and ethanol on cultured rat hepatocytes: metabolism dependent generation of reactive oxygen species as a mechanism of injury. (in preparation for submission)

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ABSTRACTS

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Schwertz, D.W. and R.G. Lamb. Effect of CCl_4 exposure on glycerolipid biosynthesis in rat hepatocytes. Fed. Proc. 38: 539, March-April, 1979.

Lamb, R.G. and W.L. Dewey. Effect of morphine on mouse liver triglyceride biosynthesis. Pharmacologist 21: 226, 1979.

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Snyder, J.W., McCue, S.B. and Lamb, R.G. Ethanol-dependent alterations in cultured hepatocyte glycerolipid metabolism. Fed. Proc. 43: 755, 1984.

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Lamb, R.G. and McGuffin, M. Phospholipase C-dependent alterations in cultured hepatocyte phospholipid metabolism. Fed. Proc. 44: 518, 1985.

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Lamb, R., Huband, S. and Bush, R. Maintenance of chemical biotransformation in primary cultures of adult rat hepatocytes. FASEB J. 7:A572, 1993.

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Huband, S. and Lamb, R. Hepatocellular phospholipases A and C are activated by oxyradicals generated by a Haber-Weiss reaction. FASEB J. 9:A957, 1995.

Kirkup, C., Humphrey, R. and Lamb, R. Influence of ethanol, acetaminophen (AAP) or both on cultured hepatocyte function. FASEB J. 9:A941, 1995.

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Kirkup, C., Humphrey, R. and Lamb, R. The cytotoxic effects of ethanol, acetaminophen or both are due in part to changes in phosphatidylcholine biosynthesis. FASEB J. 10:A797,1996.

Lamb, R.G., Willoughby,K.A., Bush-Humphrey,R., Ellis, E.F. Ethanol and strectch-induced injury act additively by an oxygen radical-depedendent mechanism to increase astrocyte phosphatidylcholine biosynthesis. Journal of Neurotrauma 15:1998.

RESEARCH SUPPORT

1) NIH Postdoctoral Training Grant Award in Clinical Pharmacology (1970-1972).

2) ASPET Travel Award to attend 6th International Pharmacology Meetings in Helsinki, Finland, July 1975.

3) A.D. Williams Medical School small grant award (1975-1976), $4,500, Medical College of Virginia.

4) Co-Investigator, NIH Grant (AM 18067), Hepatic Lipid Metabolism and Ethanol Effects (1973-1977), $261, 065, (P.I. Harold J. Fallon).

5) Co-Principal Investigator, NIH Grant (HL 16464), Synthesis and Study of Hypolipidemic Agents (1975-1977), $67,000. In collaboration with Claude Piantadosi at the University of North Carolina, Chapel Hill, N.C.

6) Principal Investigator, NIH Liver Metabolism Program Project Grant (AM 18976), Glycerolipid Metabolism in the Liver (1976-1981), $386,370.

7) Principal Investigator, NIH Grant (AM 21924), Toxins and Hepatic Glycerolipid Biosynthesis (1979-1981), $113,659.

8) Principal Investigator, NIH Liver Metabolism Program Project (AM18976), Hepatic Glycerolipid Metabolism (1981-1984), $251,670.

9) Faculty Grant-in-aid (1982-1983), $5,000, Medical College of Virginia.

10) Principal Investigator, NIH Grant (AM 31115), Toxins and Hepatocellular Phosphoglyceride Catabolism (1983-1988), $251,051.

11) Principal Investigator of Grant from the Distilled Spirits Council of the United States, Mechanisms of Alcohol-Dependent Liver Cell Injury (1985-1986), $14,340.

12) Co-Principal Investigator (with Dr. Borzelleca) of Grant from the Environmental Protection Agency, CR 812558, Investigations into a Mechanistic Approach to Predicting Interactions between Drinking Water Pollutants (1985-1988), $604,638.

13) Principal Investigator, NIH Grant (DK 33537), Regulation of Hepatic Glycerolipid Metabolism (1987-1991), $234,000.

14) Co-Principal Investigator (with Dr. Borzelleca) of Grant from the Environmental Protection Agency, CR 812558, Investigations into a mechanistic approach to predicting interactions between drinking water contaminants -- validation of the cultured hepatocyte system (1988-1990), $540,000.

15) Principal Investigator, NIH Grant (DK 31115), Toxins and Hepatocellular Phosphoglyceride Catabolism, (1988-1993), $370,000.

16) Principal Investigator, NIAAA Grant (AA08758), An in vitro model of ethanol-dependent liver cell injury, (1991-1998), $225,000 (Shannon Award).

17) Principal Investigator, A.D.Williams Award, The role of cellular phosphatidylcholine metabolism in alcohol-induced liver cell death. **Active.**

18) Principal Investigator, Phlo, Systems, Inc., Commercial development of vitamin E phosphate encapsulated in phosphatidylcholine liposomes as an orally active antioxidant and cytoprotectant. **Active.**

COOPERATIVE RESEARCH EFFORTS

1) Member of Liver Metabolism Program Project (1976-1984) Collaborators: Drs. Guzelian, Goldman, Gewirtz, Ruddy, Diegleman, Zieve, Moore, Vlahcevic, Hylemon and Fallon.

2) Member of Lipid Metabolism Program Project (1986-1991) Collaborators: Drs. Vlahcevic, Schwartz, Hylemon, Moore, Grogan, and Heuman.

3) EPA Project with Dr. Borzelleca (1985-1990).

4) Brain injury study with Dr. Kontos (J. Neurosurg. 56:695,1982).

5) Influence of morphine on hepatic triglyceride biosynthesis with Dr. Bill Dewey (J. Pharmacol. Exp. Ther. 216:496-499, 1981).

6) Effect of hydrazine on hepatic triglyceride synthesis with Dr. Bill Banks (Biochim. Biophys. Acta 574:440, 1979).

7) Response Surface Analysis of chemical interaction data in cultured rat hepatocytes with Dr. Chris Gennings (Toxicol. Appl. Pharmacol. 101: 106-113, 1989).

8) Alterations in cultured astrocyte phosphatidylcholine biosynthesis induced by cell stretching, alcohol or both. Collaboration with Dr. Earl Ellis (J. Neurochem. 8:1904-1910; 1997; J. Neurotrauma 15:1998).

RESEARCH SOCIETY MEMBERSHIPS

1) Amer. Society for Pharmacol.and Exper. Therap. (ASPET), 1976.
2) Sigma Xi, 1972.
3) Amer. Assoc. for Advancement of Science (AAAS)
4) Virginia Academy of Science
5) New York Academy of Science
6) American Gastroenterology Society
7) Drug Metabolism Society, division of ASPET

IV. SERVICE

I have not kept a record of all my service related activities within the Department, School, University and Community. Therefore, the more recent years are more complete than those of the past and dates are estimated.

DEPARTMENT (PHARMACOLOGY AND MEDICINE)

A. Comprehensive Exam Committee (1976-78, 1980-82)
B. Admissions Committee (1988 - 1990); Chair 1990
C. Medical Curriculum Committee (1988 - present)
D. Committee for Assessment of Seminar Programs (1988 - 1992)
E. Graduate Student Exam Committees (each year)
F. Toxicology Training Grant Member (1980 - 1993)
G. Gastroenterology Training Grant Member (1979 - 1992)
H. Liver Metabolism Program Project (1976-1984)
I. Lipid Metabolism Program Project (1986 - 1992)
J. Graduate Student Advisor
K. Medical Student Advisor
L. Organizer of Phospholipid Symposium (1989)

M. Minority Student Enrichment Program, Mentor (1982 - present)
N. Organizer and Coordinator of Toxicology Seminars (1989-1991)

SCHOOL

A. Promotion and Tenure Committees
B. Search Committee for Physiology Chairman (1988-89)
C. SBHS Committee on Dental Curriculum (1988 - present)
D. Dean's Representative on Comprehensive Exams
E. Forbes Day Judge and Proposal Reviewer (1988-1992)
F. Kinloch-Nelson Day Judge (1985; 1987; 1992)
G. Ad Hoc Purpose and Effectiveness Committee SBHS (1993)
H. Gerentology Subject Matter Committee (1992 - 1997)

UNIVERSITY

A. Faculty Senator (1987-90), (2000-2003)
B. Secretary and Treasurer of Faculty Senate (1989; 1990)
C. Faculty Senate Executive Committee (1989; 1990) (2000; 2001; 2002)
D. University Council Alternate (1989)
E. University Council Member (1989-1991), (2001, 2002)
F. Academic Programs and Research Committee (1988-90), (2000-2003)
G. Academic Support Services Committee (1987-88)
H. Faculty Affairs Subcommittee of University Council (1990)
I. Vice President of University Council (1991).
J. Co-Chair Academic Programs and Research Committee (Active)

COMMUNITY AND STATE

A. Advisory Committee for Gifted Students in Chesterfield County
B. Cubscout Master for Pack 892 in Chesterfield County (1975-1981).
C. Webelos Leader (1979-1981)
D. Reams Road Baseball Coach (1976-82)
E. Reams Road Basketball Coach (1979-81)
F. PTA member (1974-1993))
G. Booster Club member, Monacan High School (1980-87)
H. VA Heart Assoc. Research Grant Review Committee (1985-88).

NATIONAL AND INTERNATIONAL

A. Manuscript Reviewer:

1. Lipids
2. Biochemica Biophysica Acta
3. J. Lipid Research
4. Toxicology and Applied Pharmacology

5. Biochemical Pharmacology
6. J. Clinical Investigation
7. J. Pharmacology and Experimental Therapeutics
8. Alcoholism: Experimental and Clinical Research
9. Stroke
10. Life Sciences
11. Food and Chemical Toxicology
12. Metabolism

B. Grant Reviewer:

1. Veterans Administration
2. Environmental Protection Agency
3. American Heart Association

C. Chairman of ASPET program session

1. Halogenated Hydrocarbons, Houston, Texas (1978)
2. Alcohol, Anaheim, California (1985)
3. Halogenated Hydrocarbons, St. Louis, Missouri (1986)

D. Participant in Scientific Meetings

1. International Symposium on Drugs Affecting Lipid Metabolism, Phildelphia, Pa (1971)
2. International Congress of Fat Research, Milan, Italy (1975), Cambridge, England (1977)
3. International Pharmacology Meetings, Drugs Affecting Lipid Metabolism, Helsinki, Finland (1975)
4. NIH Workshop on Lipid Metabolism, Washington, DC (1976)
5. International Congress of Liver Diseases, Basel, Switzerland (1980)
6. 35th Falk Symposium, Liver in Metabolic Diseases, Basel, Switzerland (1982)
7. 38th Falk Symposium, Mechanisms of Hepatocyte Injury and Death, Basel, Switzerland (1983)
8. Southeastern Regional Lipid Conference, attended 8 times, Chairman of three sessions on lipid metabolism.
9. Federation of American Societies for Experimental Biology (FASEB) attended annually since 1969.
10. American Society for Pharmacology and Experimental Biology (ASEPT) attended annually since 1976; however, in recent years M II pharmacology lectures have conflicted with this meeting.
11. American Association for the Study of Liver Diseases (ASSLD), I have attended this meeting several times.
12. Gordon Conference on Lipid Metabolism (attended various times).
13. EPA Workshop on Halogenated Hydrocarbons, Cincinnati, Ohio (1988)

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

Robert G. Lamb — Group Art Unit: 1615

Application No.: 09/670,346 — Examiner: Gollamudi S. Kishore

Filed: September 27, 2000 — Atty. Dkt. No.: 021941-00001

For: VITAMIN E PHOSPHATE/PHOSPHATIDYLCHOLINE LIPOSOMES TO PROTECT FROM OR AMELIORATE CELL DAMAGE

SECOND DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Dr. Robert G. Lamb, hereby declare and state:

I am the same declarant that signed the FIRST DECLARATION UNDER 37 C.F.R. §1.132, submitted herewith.

I have performed or overseen the performance of various experiments studying the inventive Vitamin E phosphate/polyenylphosphatidylcholine (VEP/PPC) in biological assays designed to examine VEP/PPC's capability of protecting against cell damage from oxidative stress and stimulating repair of cells damaged due to oxidative stress. A summary of my findings is set forth as follows.

Oxidative stress adversely affects the cells of all human and animal tissues. Oxidative stress occurs when the cellular level of free radicals (oxidants) exceeds the

cell's antioxidant capacity. Oxidative stress causes cell injury, and oxidative stress-induced cell injury is associated with, among other things, aging, cancer, emphysema, heart disease, Alzheimer's disease, alcoholic liver disease and drug-induced tissue injury. I have conducted human cell experiments (including on liver and red blood cells) along with experiments utilizing cultured rat cells, isolated rat cells (including on liver, lung, spleen, kidney, brain and intestine cells), and intact rats. Results from these experiments indicate that human and rat cells respond in a similar manner (alterations in PC biosynthesis) to oxidative stress.

Antioxidants reduce the adverse cellular effects of oxidative stress. Vitamin E and vitamin E esters such as vitamin E phosphate (VEP), vitamin E acetate, and vitamin E succinate, are recognized antioxidant agents. However, VEP chelates calcium and forms a water insoluble complex. Therefore, it is necessary to develop a method for keeping VEP soluble in the body, since high calcium levels are present in cell medium and biological fluids. This problem was resolved by encapsulating VEP in small liposomal particles of polyenylphosphatidylcholine (PPC), as I have shown in the above-identified patent application.

Surprisingly, I found that VEP/PPC is not only a much more potent antioxidant and cytoprotectant than Vitamin E or ester derivatives thereof, but that VEP/PPC can actually reverse (repair) cell damage. VEP/PPC has the unique ability to markedly reduce the adverse cellular effects of oxidative stress in the cells of all human tissues because VEP/PPC targets cell membranes, scavenges free radicals and increases the cell membrane repair function. A key to any agent's effectiveness is its delivery, and

the inventive liposomal delivery system enables VEP/PPC to cross cellular membranes and rapidly distribute to target tissues.

Materials and Methods

Primary cultures of rat hepatocytes (Lamb et al., 1994) and rat astrocytes (Lamb et al., 1997) have been described in detail in the referenced articles.

All methods used to measure cell viability, lipid peroxidation, cellular phosphatidylcholine (PC) biosynthesis and enzyme activity have also been presented in detail in Lamb, et al., 1994; 1997. All assays were performed in triplicate and experiments were repeated in at least two different cell preparations. Experimental data is expressed as percent of control ± standard error of the mean (SEM). Statistical analysis was performed by Student's two-tailed t test or one way analysis of variance (ANOVA). If the experimental data is significantly different from controls ($p \leq 0.05$), this is indicated in the Figure or Table by an asterisk or letter.

All materials are commercially available from Sigma Chemical Company. Additions to cells were made at 24 hour (h) intervals. Cells were cultured in Waymouth 752/1 media supplemented with insulin, dexamethasone, vitamin E, testosterone, estradiol and arachidonic acid. VEP, PPC and VEP/PPC were sonicated in deionized water and sterilized before they were added to the cell media.

Summary and Conclusions

A. The PC Biosynthesis Model For Determining Oxidative Stress

The involvement of free radicals in oxidative stress-induced cell injury has been proposed for many years. However, determining the validity of the free radical theory of cell injury is difficult since free radicals are very reactive and short-lived chemical entities. Therefore, it is essential to develop techniques which can detect the formation of free radicals and the effects of free radicals on cell functions.

I have developed a process for determining oxidative stress in intact cells, which is the subject of a U.S. patent granted to me (U.S. Patent No. 6,218,130, issued April 17, 2001, entitled "TEST FOR OXIDATIVE STRESS USING CELL SUSPENSIONS"). My test for oxidative stress is based on the observation that the polyunsaturated fatty acids (PUFAs) of membrane phospholipids such as PC, the major phospholipid of all cellular membranes, are excellent free radical traps. The cellular membranes are injured when the PUFAs trap free radicals, and such injury increases (during a period of ≤ 24 hours (short-term exposure), see pages 5-7 hereof) cellular PC biosynthesis (Figure 1, reaction 3). Therefore, the determination of free radical-dependent changes in cellular PC biosynthesis is an excellent model for determining oxidative stress.

Most membranes of mammalian cells contain a phospholipid bilayer in which are imbedded P450-dependent monooxygenases. The P450-dependent metabolism of various agents such as fatty acids, steroids, ethanol, acetaminophen (Tylenol), and other oxidative stressors results in the formation of reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) (Figure 1, reaction 1). Hydrogen peroxide and superoxide anion interact with redox-active iron (Haber-Weiss Reaction) to produce the very toxic hydroxyl radical [•OH] (Figure 1, reaction 2). It is impossible for the hydroxyl radical to move far from its site of formation (P450 monooxygenase), so

the hydroxyl radical must interact with the PUFAs of membrane associated PC (Figure 1, reaction 3). Radicalized membrane PC (membrane injury) disrupts essential membrane functions such as signal transduction, enzyme activity and chemical transport. Therefore, the cell must have an efficient membrane repair system (involving phospholipid metabolism) to repair free radical-dependent membrane injury. Otherwise, the free radical-induced cell injury can result in aging, drug-dependent tissue damage, and various diseases such as cancer, emphysema, heart disease, Alzheimer's disease, and alcoholic liver disease.

Most cells contain cytosolic enzymes, such as phospholipases and phosphocholine cytidyltransferase, that can rapidly translocate onto and off of membranes as needed, to rapidly repair injured membranes by increasing PC hydrolysis and PC biosynthesis (Lamb, et al., 1988). Moderate levels of membrane injury are rapidly reversed and repaired; however, high levels of membrane injury result in irreversible injury and cell death (Figure 1, reaction 3).

The sequence of reactions outlined in Figure 1 is consistent with the effects of agent-induced oxidative stress on PC biosynthesis in primary cultures of adult rat hepatocytes (Figure 2). Cultured hepatocytes incubated (0 to 72 h) with 100 mM ethanol (an oxidative stressor) exhibit incubation time-dependent changes in PC biosynthesis. Short-term ethanol exposure ($\leq$ 24 h) produces an increase in PC biosynthesis (reversible injury). Long-term ($\geq$ 48 h) incubation of cells with ethanol results in a decrease in PC biosynthesis (irreversible injury). Incubation of cells with other oxidative stressors such as acetaminophen (Tylenol) (Figure 5) and iron (data not

shown herein) produce changes in cellular PC biosynthesis that are similar to those presented in Figure 2.

Figures 3 and 4 show the ethanol-dependent increase in cellular PC biosynthesis with short-term (90 min) exposure (Figure 3, black bar), and decrease in cellular PC biosynthesis with long-term (72 h) exposure (Figure 4, black bar), as compared to control (white bars). Figures 3 and 4 also show that changes in cellular PC biosynthesis were reduced by various cytoprotective agents (4-methyl pyrazole (4-MP), superoxide dismutase (SOD), catalase (CAT), deferoxamine (DEF), dimethyl sulfoxide (DMSO), VEP and PPC) which prevent free-radical induced injury by intervening at various points in the Haber-Weiss Reaction which produces hydroxyl radicals (outlined in Figure 1, reaction 2, and summarized on page 4 hereof). 4-MP inhibits P450-dependent monooxygenases; SOD is a superoxide scavenger; CAT is a hydrogen peroxide scavenger; DEF is an iron chelator; and DMSO, VEP, and PPC are hydroxyl radical scavengers. Therefore, the results set forth in Figures 3 and 4 suggest that ethanol and other oxidative stressors alter cellular PC biosynthesis as a result of a hydroxyl radical-dependent membrane injury (Haber- Weiss Reaction, Figure 1, reactions 2 and 3). The inventive VEP/PPC is successful in preventing the sequence of reactions that creates hydroxyl radicals (the common element leading to cell injury and disease from oxidative stress) since the information set forth herein and in the referenced patent application demonstrates that VEP/PPC is exceedingly effective (much more effective than other antioxidant agents) in reducing the changes in cellular PC biosynthesis which occur as a result of short-term and long-term exposure to hydroxyl radicals (oxidative stress).

Primary cultures of human liver cells incubated with 100 mM ethanol (ETOH), 1 mM acetaminophen (APAP) or both also exhibit short-term (24 h) increases and long-term (48 h) decreases in cellular PC biosynthesis, respectively (Figure 5). Other tissues (lung, spleen, kidney, brain, intestine, and erythrocyte) incubated 30 minutes with 1 mM acetaminophen also exhibit significant increases in cellular PC biosynthesis (Figure 6). These results (Figures 2-6) suggest that oxidative stress in various tissues (both human and rat) produce free radicals that rapidly injure cellular membranes by interacting with PUFAs of membrane PC. Thus, by measuring PC biosynthesis one can determine the level of cellular injury or repair of damage from oxidative stress. In other words, one can utilize the rate of PC biosynthesis to assess the formation of free radicals and the effects of free radicals on cell functions in the intact cell (Lamb, et al., 1994, 1997, and U.S. Patent No. 6,218,130 granted to Lamb).

Agent-dependent changes in cellular PC biosynthesis represent the cell's response to free radical-induced cell injury. Membrane injury is reversible when cellular PC biosynthesis is increased. As shown above, this occurs during short-term agent exposure. Irreversible cell injury and cell death occur when cellular PC biosynthesis is decreased (during long-term agent exposure). Therefore, free radical dependent membrane injury is or is not reversible, depending on whether the membrane's repair rate (PC biosynthesis) is greater than or equal to the rate of membrane injury. There is a critical balance between the rate of cell injury and the cell's membrane repair rate. If one could increase the cell's membrane repair rate (increase PC biosynthesis), decrease the rate of free radical-dependent cell injury (by using a cytoprotective agent) or both, then one would reduce the adverse cellular effects of oxidative stress.

B. Comparative Studies Involving the Inventive VEP/PPC

In order to show the inventive attributes of VEP/PPC, I have compared the effects of vitamin E, various ester derivatives of vitamin E (vitamin E acetate (VEA), vitamin E succinate (VES) and VEP), PPC, and VEP/PPC on oxidative stress-dependent changes in cellular PC biosynthesis and lipid peroxidation. As shown with the various cytoprotective agents of Figures 3 and 4, a potent cytoprotective agent reduces the oxidative stress-dependent increases in cellular PC biosynthesis with short-term exposure to oxidative stressors, and reduces the oxidative stress-dependent decreases in cellular PC biosynthesis with long-term exposure to oxidative stressors. The data presented below shows that VEP/PPC is such a potent cytoprotective agent.

As primary cultures of adult rat hepatocytes age in culture (long-term exposure), there is a significant increase in lipid peroxidation (oxidative stress) and a decrease in PC biosynthesis. Table 1 shows that this occurs even though 25 μM vitamin E is present in the culture media (Table 1, without VEP). The addition of VEP (25 μM) markedly reduced the age-dependent increase in lipid peroxidation and decrease in PC biosynthesis (oxidative stress).

Table 1
Alterations in lipid peroxidation and PC biosynthesis of cultured liver cells incubated in culture media containing 25 μM vitamin E, with or without 25 μM VEP.

	Percent of Control ± SEM			
	Peroxidation		PC Biosynthesis	
Days	Without VEP	with VEP	without VEP	with VEP
0	100 ± 7	100 ± 21	100 ± 4	100 ± 4
1	755 ± 132***	89 ± 18	34 ± 1***	83 ± 6
2	687 ± 23***	43 ± 4	50 ± 2***	89 ± 6
3	842 ± 32***	75 ± 14	23± 1***	94 ± 5

*** Significance from control is $p \leq 0.01$ (0 days)

The dose-dependent effect of VEP and VES on the age-dependent (24 h) decrease in cellular PC biosynthesis is shown in Figure 7. A 100 μM dose of VEP and VES increase cellular PC biosynthesis six-fold and two-fold, respectively (Figures 7 and 8). Similar doses of VEA and vitamin E (indicated as CONTROL or CTRL in the Figures) have no effect on cellular PC biosynthesis or phosphocholine cytidylyltransferase (PCT) activity (the rate-limiting enzyme in PC biosynthesis) (Figure 8). VEP and VES also increase cellular PCT activity six-fold and two-fold, respectively (Figure 8).

These results (Table 1, Figures 7 and 8) indicate that VEP:

- stimulates the cell's membrane repair rate by increasing cellular PC biosynthesis; and
- is a better cellular antioxidant than vitamin E, VEA or VES.

The antioxidant effect of various doses (0 to 1000 μM) of vitamin E and VEP were determined. As shown in Figure 9, VEP reduced the tert-butyl hydroperoxide [TBH]-dependent peroxidation of cellular phospholipids at least five-fold more than vitamin E. VEP is a better cellular antioxidant than vitamin E because VEP targets the membrane's phospholipid bilayer and has the unique ability to scavenge free radicals. Additionally, VEP increases the cell's membrane repair rate by increasing PC biosynthesis, thereby repairing damage which has already occurred. As a result, VEP has the unique ability to reduce and reverse the adverse cellular effects of oxidative stress.

The antioxidant potential of VEP, PPC and VEP/PPC was then determined in isolated liver cells. As shown in Table 2, VEP, PPC and VEP/PPC reduced the oxidative stress (as measured by an increase in lipid peroxidation and PC biosynthesis) produced in liver cells by a 30 minute incubation (short-term exposure) with 1 mM ferrous iron. The antioxidant effect of VEP/PPC was greater than that of VEP, and the antioxidant effect of VEP was greater than that of PPC.

Table 2
The use of VEP (50μM), PPC (100μM) and VEP/PPC (50 μM/100 μM) to reduce the oxidative stress produced in liver cells by iron.

	Percent of Control ± SEM	
Additions	**Lipid Peroxidation**	**PC Biosynthesis**
None	100 ± 2	100 ± 3
1 mM Iron	357 ± 12a	254 ± 9a
1 mM Iron + PPC	281 ± 4b	167 ± 4b
1 mM Iron + VEP	167 ± 3bc	145 ± 4bc
1 mM Iron + VEP/PPC	124 ± 2bcd	117 ± 4bcd

a Level of significance from control [none] is $p \leq 0.05$
b level of significance from iron is $p \leq 0.05$
c level of significance from iron + PPC is $p \leq 0.05$
d level of significance from iron + VEP is $p \leq 0.05$

VEP encapsulated in PPC is at least ten times (and as much as 250 times) more cytoprotective (Figures 10-13 (1-10 μM VEP is "optimal", i.e., the level at which the maximum effect is reached and additional improvement is marginal)) than VEP which is not encapsulated in PPC (Figures 7 and 9 (100-250 μM VEP is optimal)). Note that Table 2 does not demonstrate the magnitude of the superiority in effectiveness of VEP/PPC over VEP and PPC alone, as the tests (the results of which are set forth in Table 2) were conducted over a period of only 30 minutes. Additionally, although VEP is cytoprotective and stimulates cell repair in vitro, it is only effective in vivo in the form of VEP/PPC because, as discussed above, VEP alone chelates calcium and forms a water insoluble complex.

Figure 10 also shows the antioxidant effect of VEP/PPC. This figure shows the results of various levels of VEP/PPC on PC biosynthesis. The dose expressed in Figures 10-13 represents the dose of VEP, and the dose of PPC is always twice the dose of VEP. Therefore, a dose of 2.5 μM VEP/PC in these Figures represents 2.5 μM VEP and 5 μM PPC. VEP/PPC produced a dose-dependent decrease in the ethanol-dependent increase in cellular PC biosynthesis with short-term exposure (24 h) to 25 mM ethanol. The ethanol-induced increase in cellular PC biosynthesis (150 percent of control) was completely blocked by 10 μM VEP/PPC. A 1 μM dose of VEP/PPC reduced the ethanol-dependent increase in oxidative stress (increase in PC biosynthesis) by more than 50%.

Figure 11 shows that various doses of VEP/PPC also reduced the ethanol-dependent decrease in cellular PC biosynthesis with long-term exposure (48 h) to 25 mM ethanol. The ethanol-dependent, 50% decrease in cellular PC biosynthesis was completely blocked by 1 μM VEP/PPC.

Figure 12 shows that VEP/PPC also had a dose-dependent effect on the adverse cellular effects of long-term (72 h) exposure to 1 mM acetaminophen (Tylenol). The acetaminophen-dependent decrease in cellular PC biosynthesis was reduced by more than 50% by 1 μM VEP/PPC.

Figure 13 shows that the age-dependent decrease (24 h) in cellular PC biosynthesis is also reduced by various doses of VEP/PPC. A 1 μM dose of VEP/PPC produced a 30% increase in cellular PC biosynthesis.

VEP/PPC (25 μM VEP/50 μM PPC) also reversed the adverse cellular effects of long-term ethanol exposure, as shown in Table 3. Cellular PC biosynthesis was

significantly reduced after 96 hours of exposure to 25mM or 50 mM ethanol. However, in the event where VEP/PPC (25 µM VEP/50 µM PPC) was added for a period of 24 hours after cells had been exposed to ethanol for 72 hours, VEP/PPC completely reversed the adverse cellular effects of 25 mM ethanol (increased cellular PC biosynthesis to control levels) by the end of the 24-hour period of VEP/PPC exposure and significantly increased cellular PC biosynthesis in cells exposed to 50 mM ethanol during the same period.

Table 3
The use of VEP/PPC to effect reversal of damage from ethanol on cultured liver cells.

Additions (exposure time)	**Percent of Control ± SEM**
None (96 h)	101 ± 6
25 mM ethanol (96 h)	38 ± 2**
50 mM ethanol (96 h)	22 ± 1**
25 mM ethanol (96 h) + VEP/PPC (24 h)	101 ± 8***
50 mM ethanol (96 h) + VEP/PPC (24 h)	68 ± 3***

** Level of significance from control (none) is $p \leq 0.05$
*** Level of significance from ethanol exposed cells is $p \leq 0.05$

The data shown herein proves that VEP/PPC is a very potent cytoprotectant (Figures 10-13 and Tables 2-3), since VEP/PPC targets cellular membranes, scavenges free radicals and stimulates the cell's membrane repair process (increases cellular PC biosynthesis). Additionally, VEP encapsulated in PPC does not chelate calcium and is at least ten times (and as much as 250 times) more cytoprotective than unencapsulated VEP (Figures 7-13).

Declaration under 37 C.F.R. §1.132
U.S. Application No. 09/670,346

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: 10/21/02

Robert Lamb

Dr. Robert G. Lamb

Figure Legends

Figure 1 - The reactions involved in the PC metabolism pathway of free radical-induced cell injury. Reactive oxygen species ($O_2^{\bullet -}$ and H_2O_2) and acetaldehyde are produced by the metabolism of ethanol by alcohol dehydrogenase (ADH) and cytochrome P4502E1 (CYP2E1) [Phase I]. CYP2EI can also metabolize other agents such as acetaminophen (Tylenol). Glutathione (GSH) is depleted by GSH peroxidase and conjugation with acetaldehyde. Hydroxyl radicals (•OH) are rapidly formed by a Haber-Weiss Reaction [Phase 2]. Hydroxyl radicals produce cell injury by rapidly interacting with the PUFAs of membrane PC. Membrane damage is repaired if PC hydrolysis and biosynthesis are rapidly increased (reversible injury) [Phase 3]. Cell death occurs when the rate of cell injury exceeds the membrane's repair capacity (irreversible injury) [Phase 3].

Figure 2 - Changes in the incorporation of labeled choline into PC (cellular PC biosynthesis) of hepatocyte monolayers incubated (0 to 72 h) with 100 mM ethanol. Each determination represents the mean ± SEM of 3 to 9 experimental values.

Figure 3 - Alterations in cellular PC biosynthesis (choline incorporation) of cells incubated 90 minutes with 100 mM ethanol alone or in combination with 0.5 mM 4-MP, 200 units of SOD, 800 units of CAT, 2 mM DEF or 0.08% DMSO. All data is expressed as a mean ± SEM of 3 to 9 experimental values. One asterisk (*) indicates that values are significantly different ($p < 0.05$) from control. Two asterisks (**) indicate that values are significantly different ($p < 0.05$) from ethanol exposed cells.

Figure 4 - Changes in cellular PC biosynthesis (choline incorporation) of cultured hepatocytes incubated 72 hours with 100 mM ethanol alone or in combination with 0.5 mM 4-MP, 200 units of SOD, 800 units of CAT, 2 mM DEF, 0.08% DMSO, 25 µM VEP, or 100 µM PPC. All values are expressed as a mean ± SEM of 3 to 9 experimental values. An asterisk indicates that values are significantly different ($p \leq 0.05$) from control.

Figure 5 - Alterations in cellular PC biosynthesis (choline incorporation) of cultured human hepatocytes incubated 24 hours and 48 hours with 1 mM acetaminophen (APAP), 100 mM ethanol (ETOH) or both (ETOH + APAP). See legend of Figure 4 for additional information.

Figure 6 - Changes in cellular PC biosynthesis (choline incorporation) of cells isolated from various rat tissues incubated 30 minutes with 1 mM acetaminophen (Tylenol). See legend of Figure 4 for additional information.

Figure 7 - Alterations in cellular PC biosynthesis (choline incorporation) of cultured hepatocytes incubated 24 hours with various concentrations (0 to 500

μM) of vitamin E phosphate (VEP) or vitamin E succinate (VES). See legend of Figure 2 for additional information.

Figure 8 - Changes in cellular PC biosynthesis (choline incorporation) and PCT activity of cultured hepatocytes incubated 24 hours with 100 μM vitamin E (CTRL), 100 μM VEA, 100 μM VES or 100 μM VEP. See legend of Figure 4 for additional information.

Figure 9 - Alterations in lipid peroxidation of liver cells incubated 20 minutes with 1 mM TBH in the presence of various concentrations (0 to 1000 μM) of VE or VEP. Each value represents the mean nmoles of malondialdehyde formed per mg of cellular protein ± SEM of three experimental values.

Figure 10 - The effect of various VEP/PPC concentrations (0 to 10 μM) on the increase in cellular PC biosynthesis (choline incorporation) produced by incubating cultured hepatocytes 24 hours with 25 mM ethanol (short-term ethanol exposure). Concentration expressed as "μM VEP/PC" represents the dose of VEP, and the dose of PPC is always twice the dose of VEP. Therefore, a concentration of 5 μM VEP/PC represents a 5 μM dose of VEP and a 10 μM dose of PPC. See the legend of Figure 2 for additional information.

Figure 11 - The effect of various levels of VEP/PPC on the decrease in cellular PC biosynthesis (choline incorporation) produced by incubating cultured hepatocytes 48 hours with 25 μM ethanol (long-term ethanol exposure). Concentration expressed as "μM VEP/PC" represents the dose of VEP, and the dose of PPC is always twice the dose of VEP. Therefore, a concentration of 5 μM VEP/PC represents a 5 μM dose of VEP and a 10 μM dose of PPC. See the legend of Figure 2 for additional information.

Figure 12 - The effect of various concentrations of VEP/PPC on the decrease in cellular PC biosynthesis (choline incorporation) produced by incubating cultured hepatocytes 72 hours with 1 mM acetaminophen (Tylenol). Concentration expressed as "μM VEP/PC" represents the dose of VEP, and the dose of PPC is always twice the dose of VEP. Therefore, a concentration of 5 μM VEP/PC represents a 5 μM dose of VEP and a 10 μM dose of PPC. See the legend of Figure 2 for additional information.

Figure 13 - Increases in cellular PC biosynthesis (choline incorporation) of liver cells incubated 24 hours with various concentrations of VEP/PPC. Concentration expressed as "μM VEP/PC" represents the dose of VEP, and the dose of PPC is always twice the dose of VEP. Therefore, a concentration of 5 μM VEP/PC represents a 5 μM dose of VEP and a 10 μM dose of PPC. See legend of Figure 2 for additional information.

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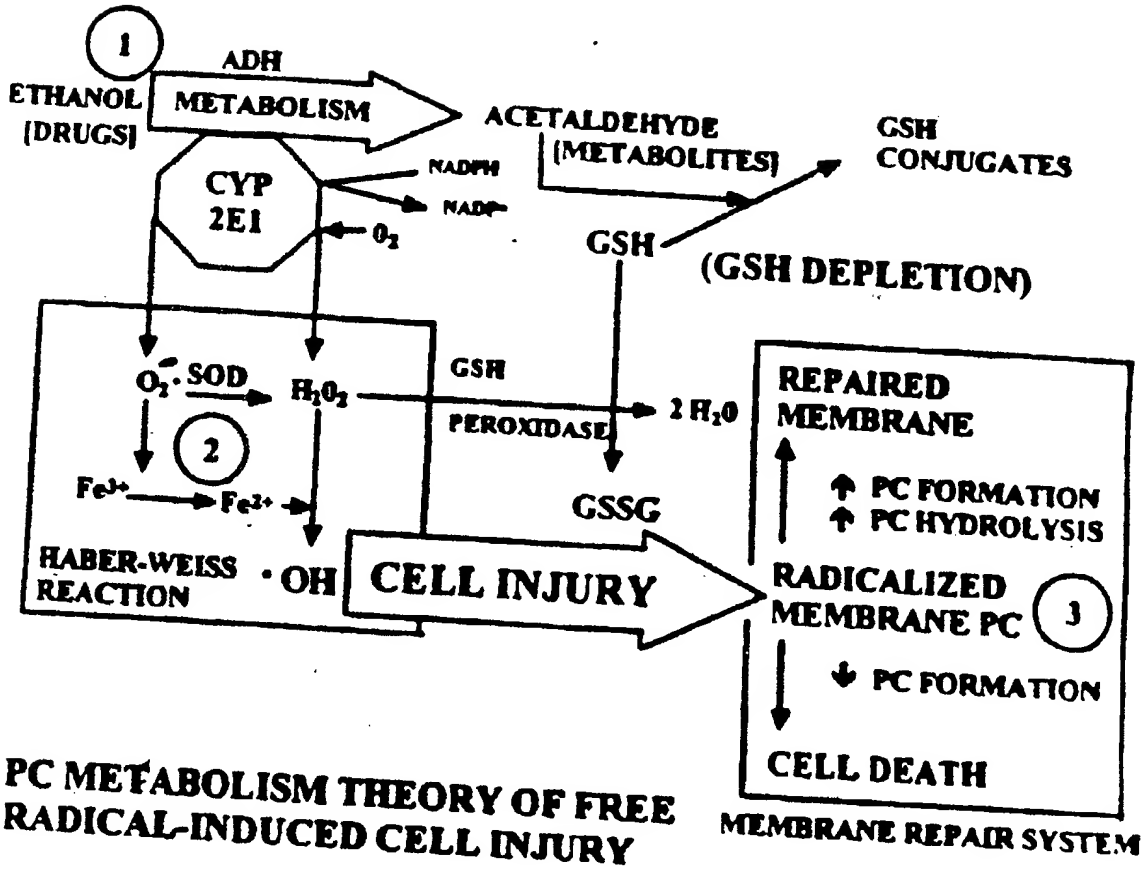
FIGURE 1



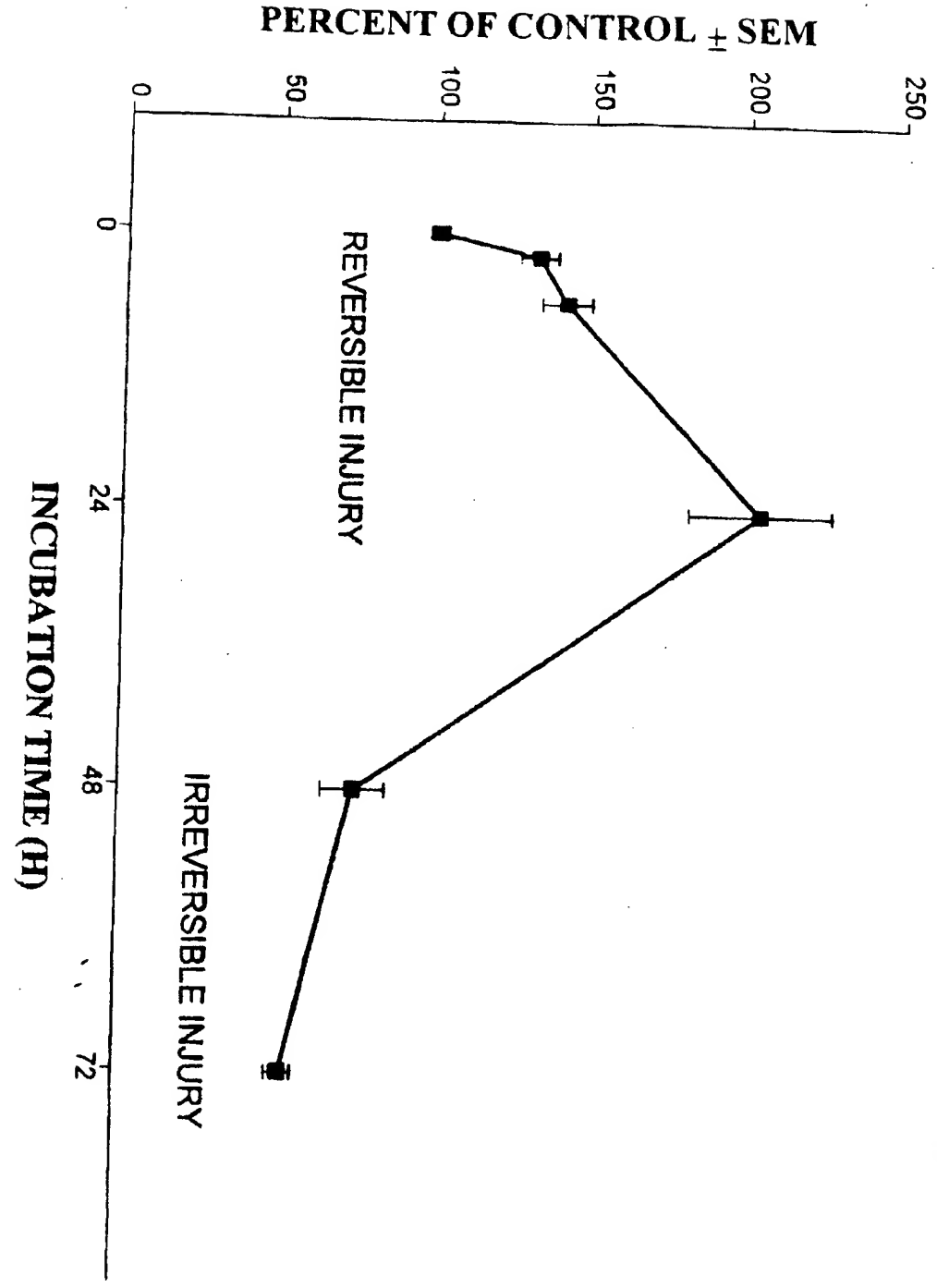


FIGURE 2

FIGURE 3

Alterations in PC biosynthesis when hepatocytes are incubated 90 min with ethanol alone or in combination with various agents.

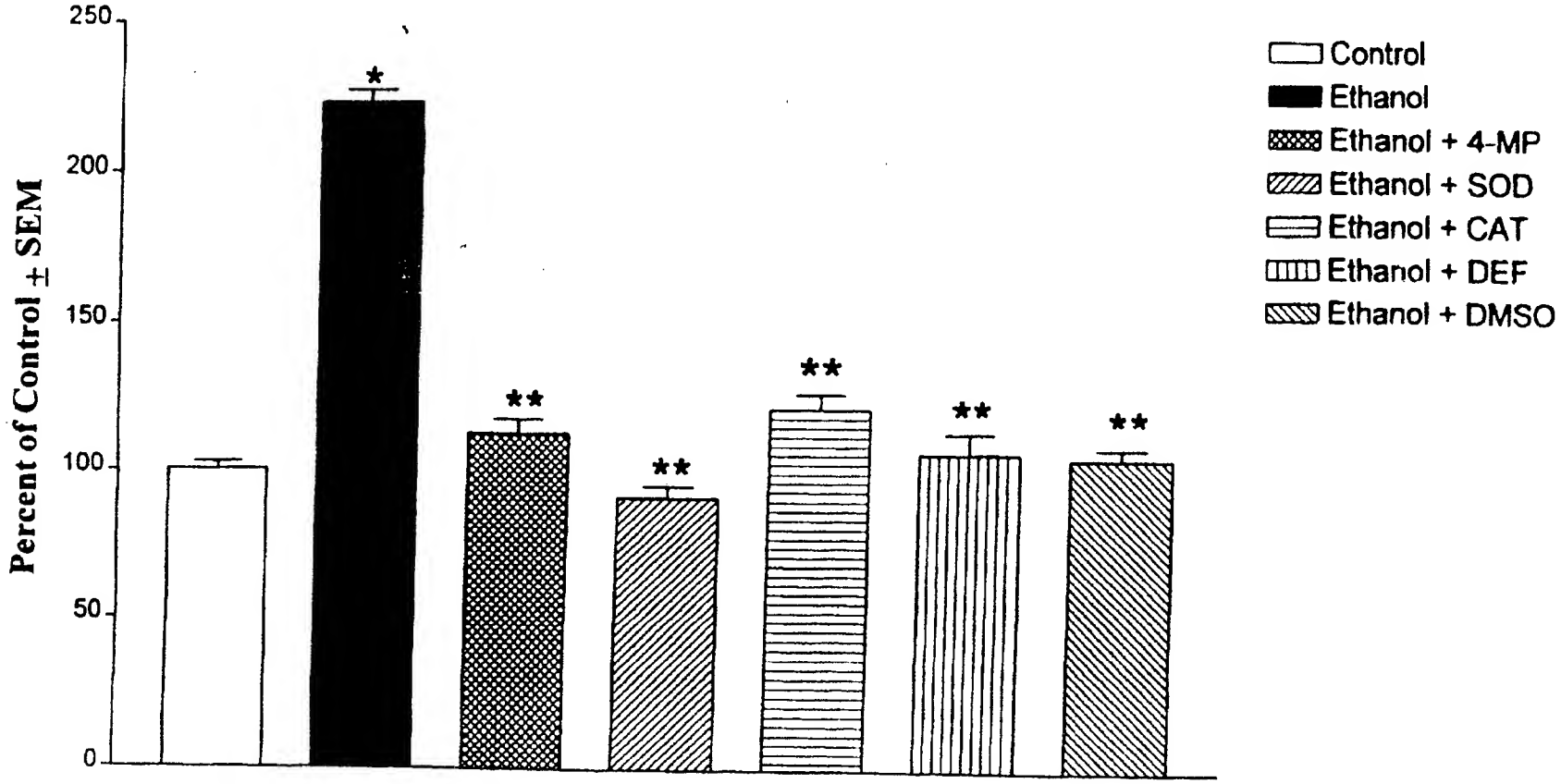


FIGURE 4

Alterations in PC biosynthesis when cultured hepatocytes are incubated 72 h with ethanol alone or in combination with other agents

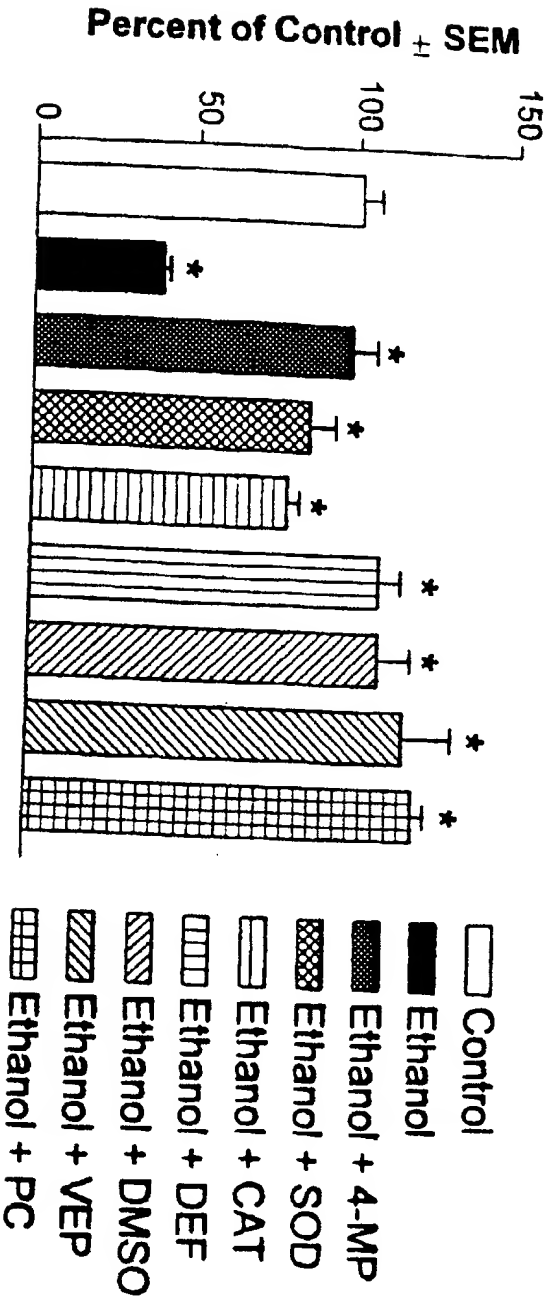
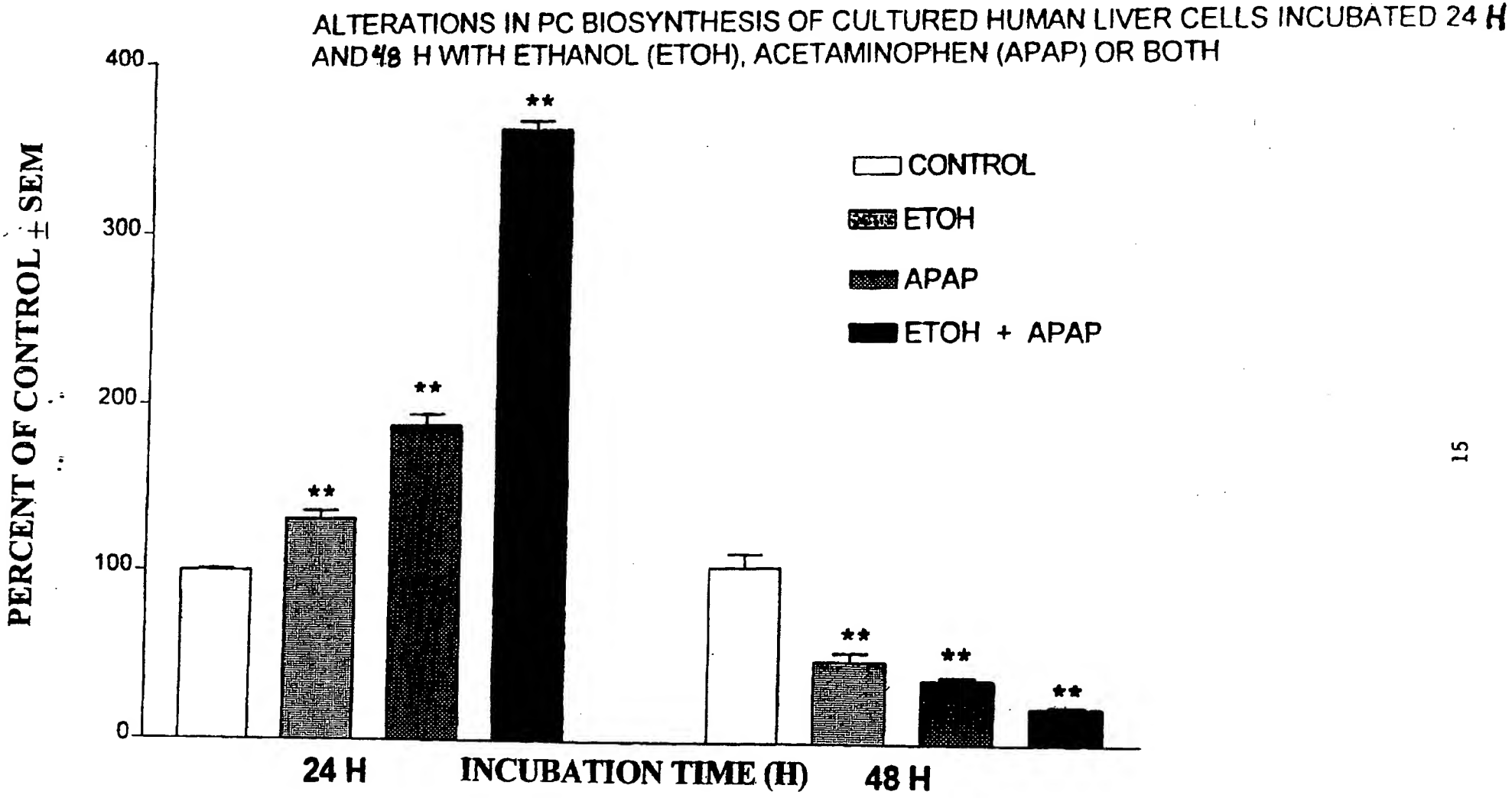
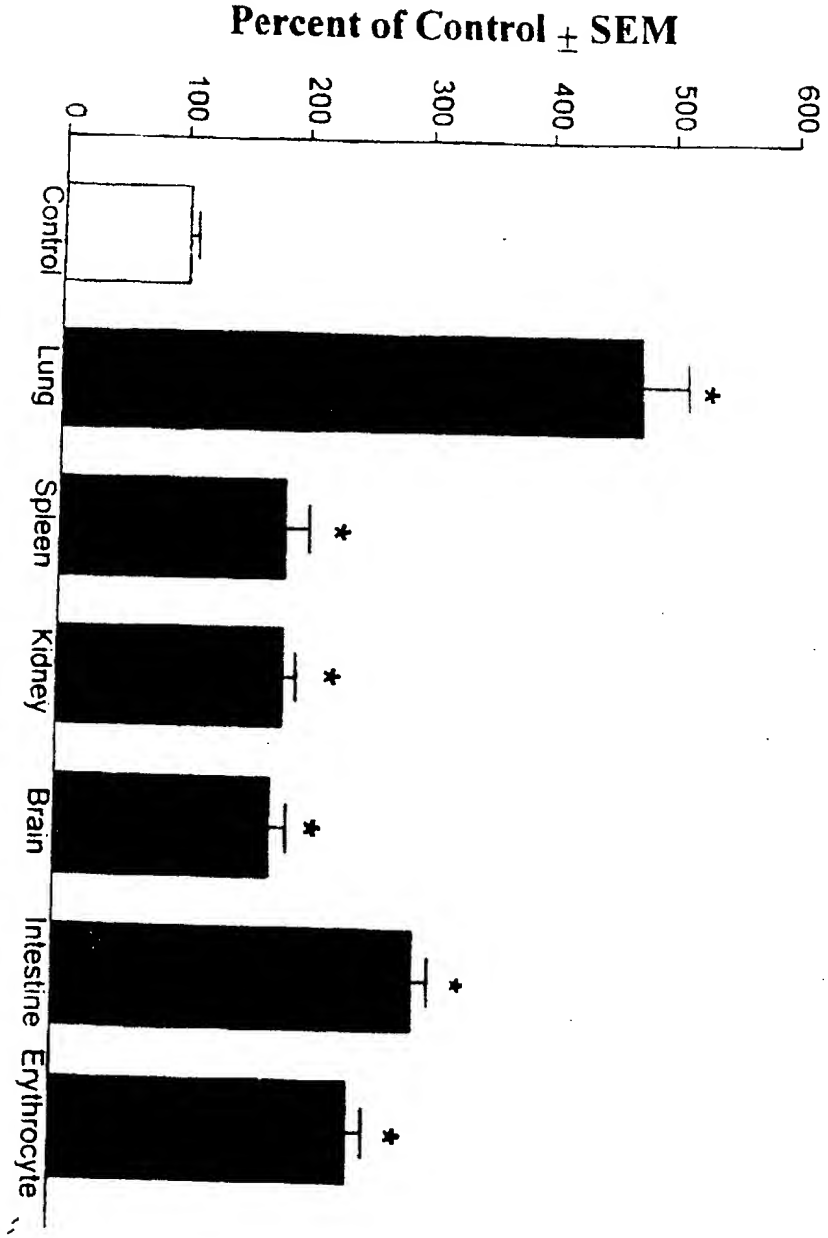


FIGURE 5





Alterations in cellular PC biosynthesis induced by a 30 min incubation with 1 mM acetaminophen in vitro

FIGURE 6

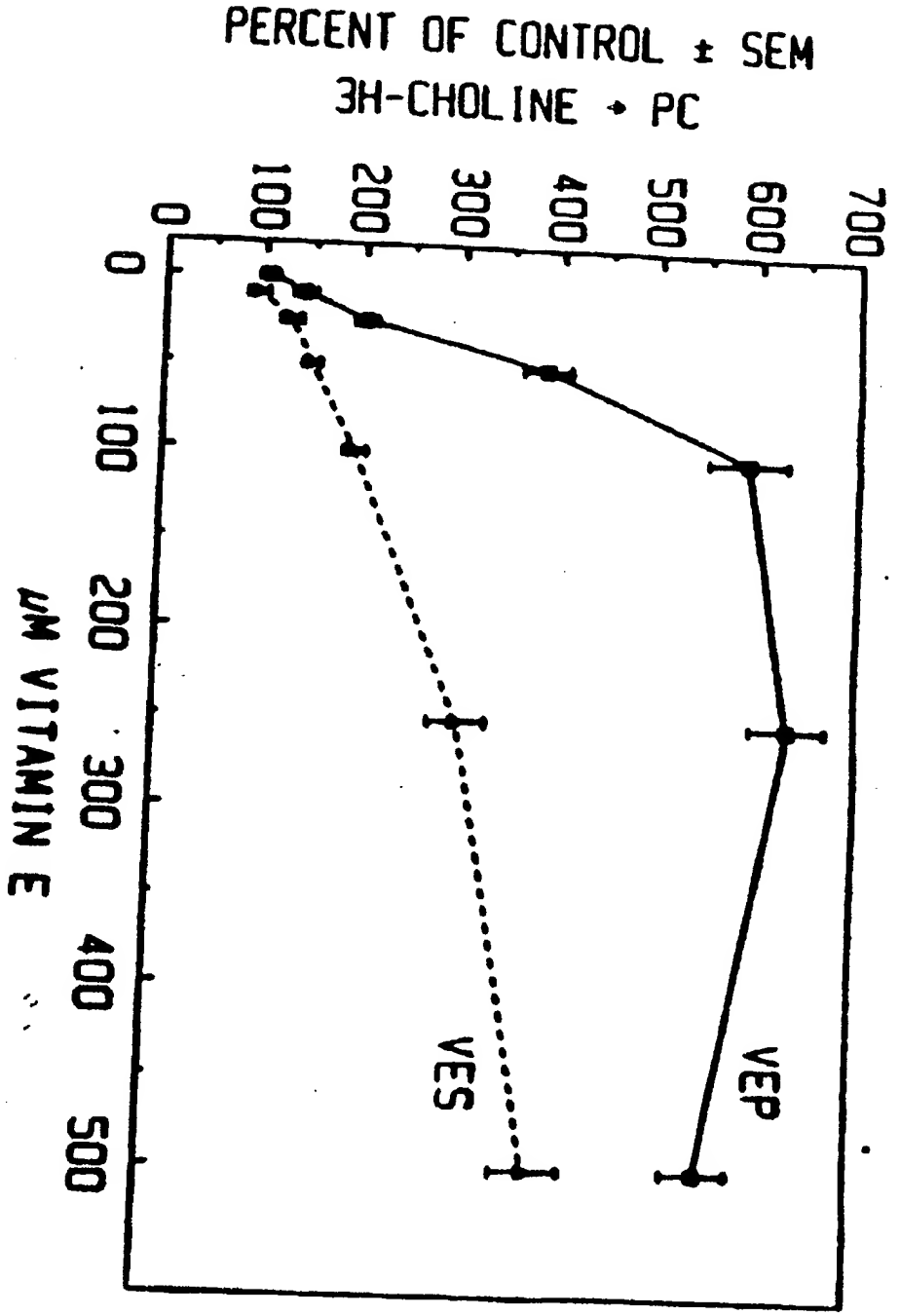


FIGURE 7

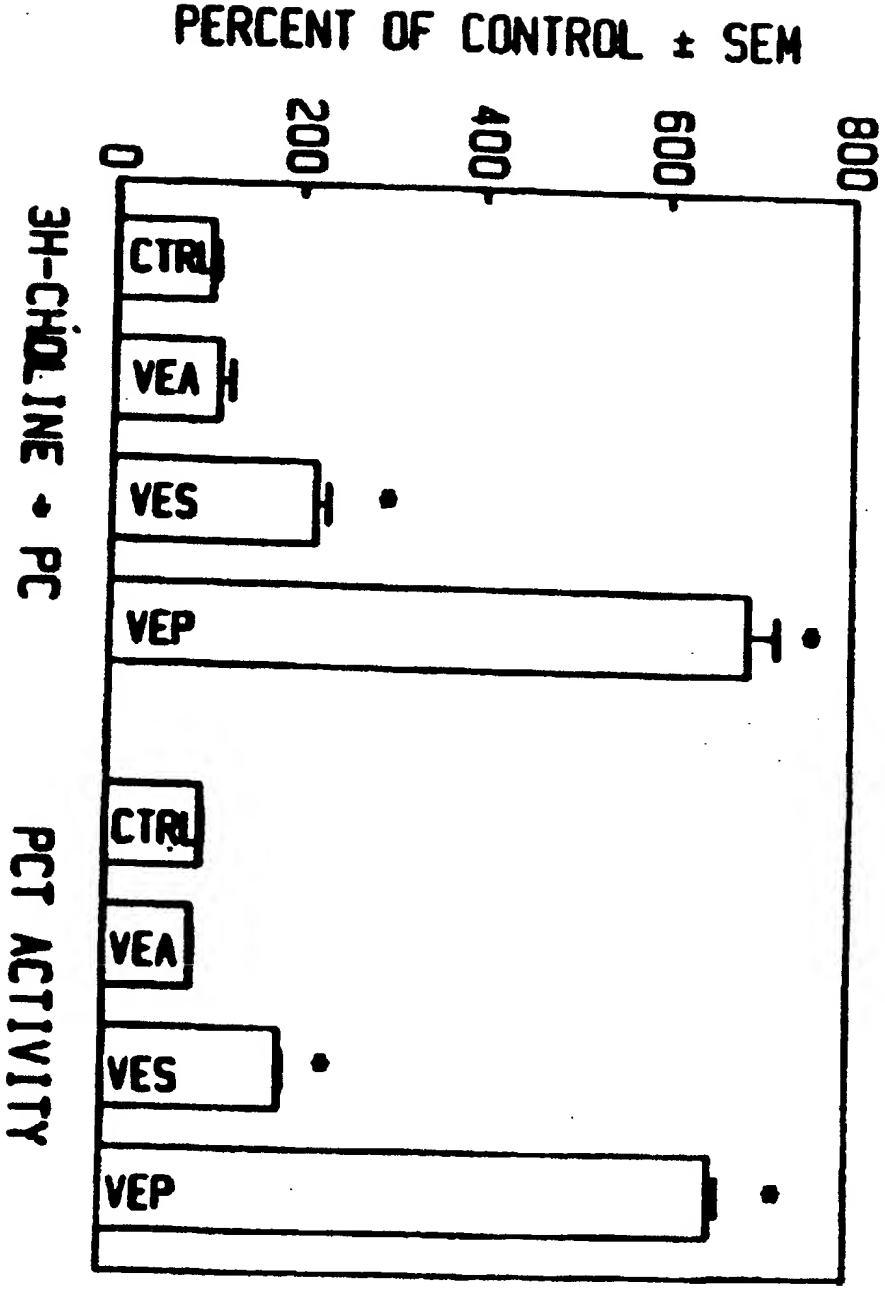


FIGURE 8

FIGURE 9

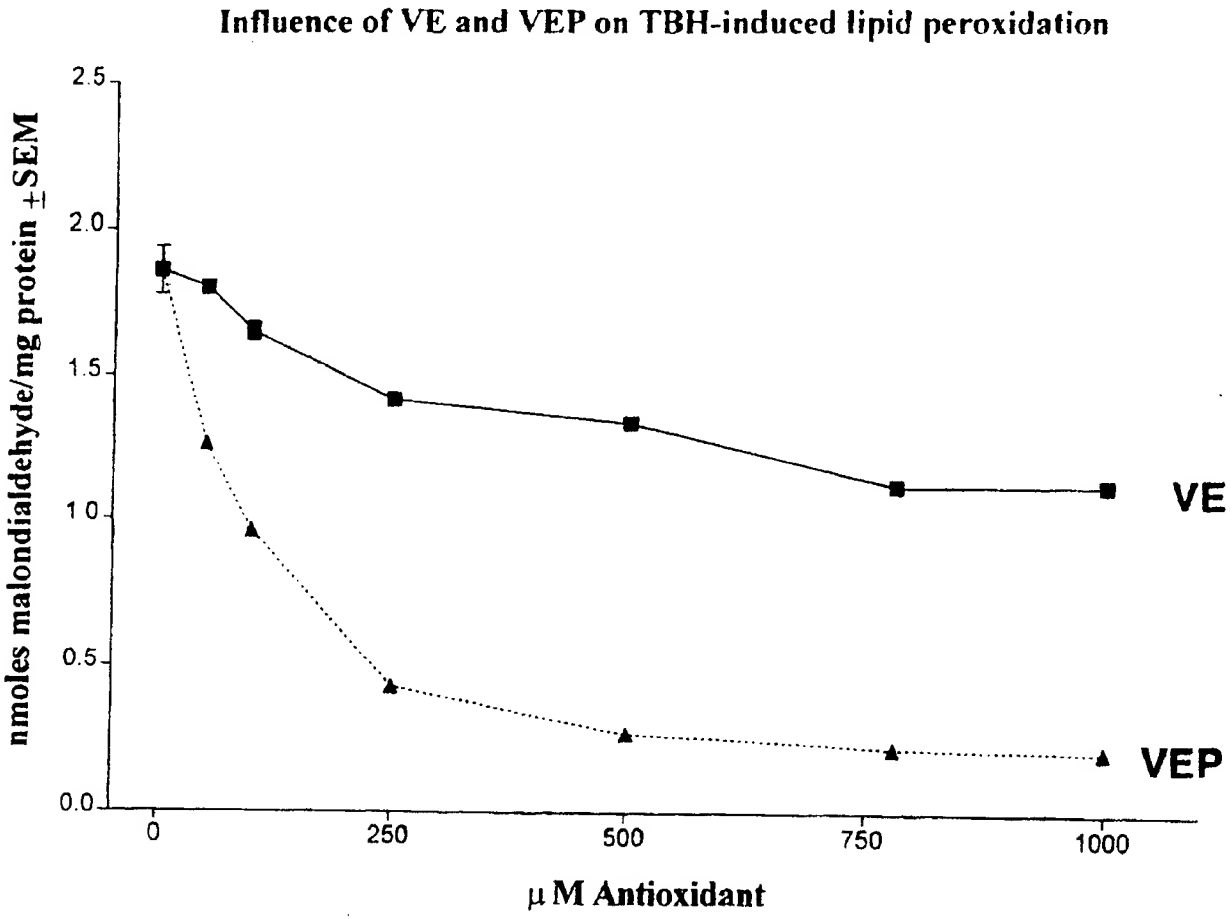


FIGURE 10

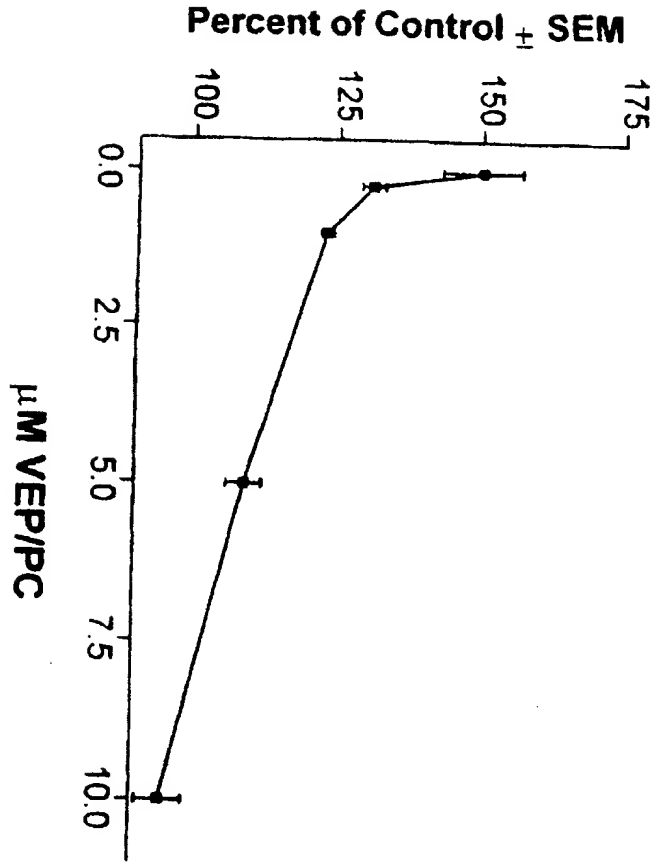


FIGURE 11

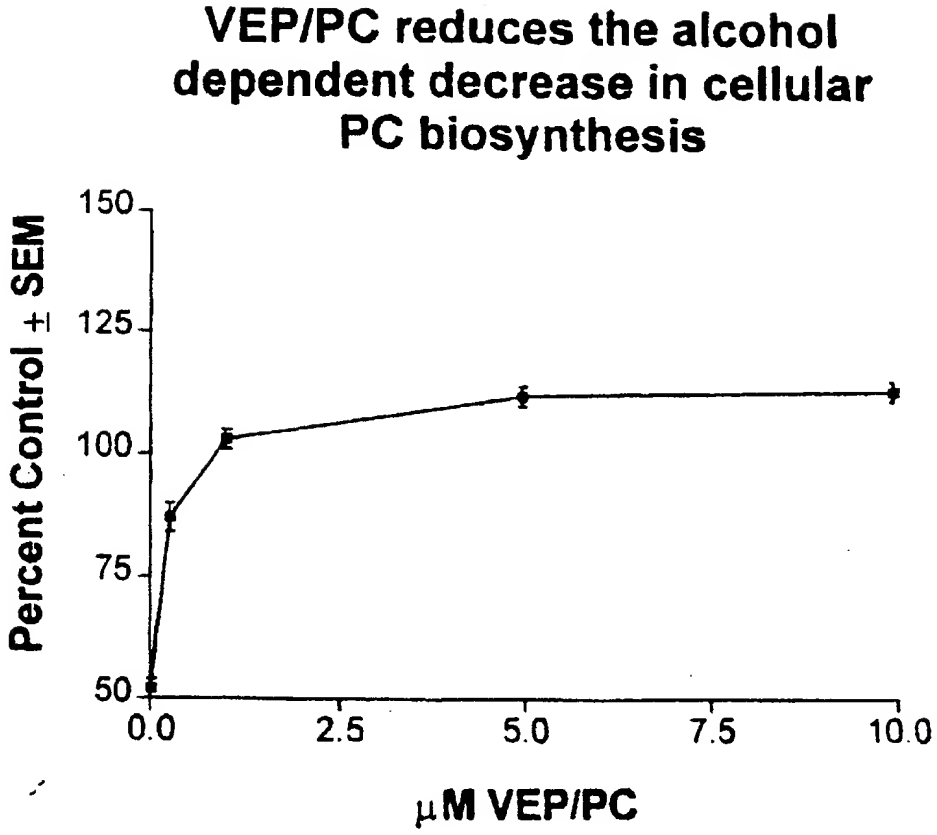


FIGURE 12

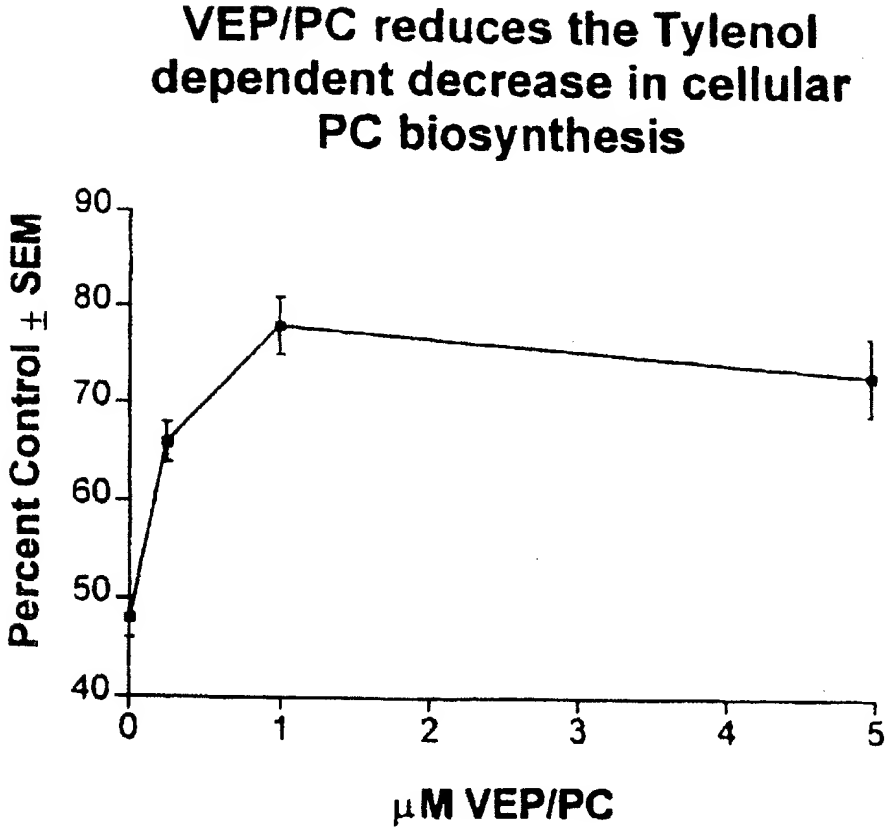
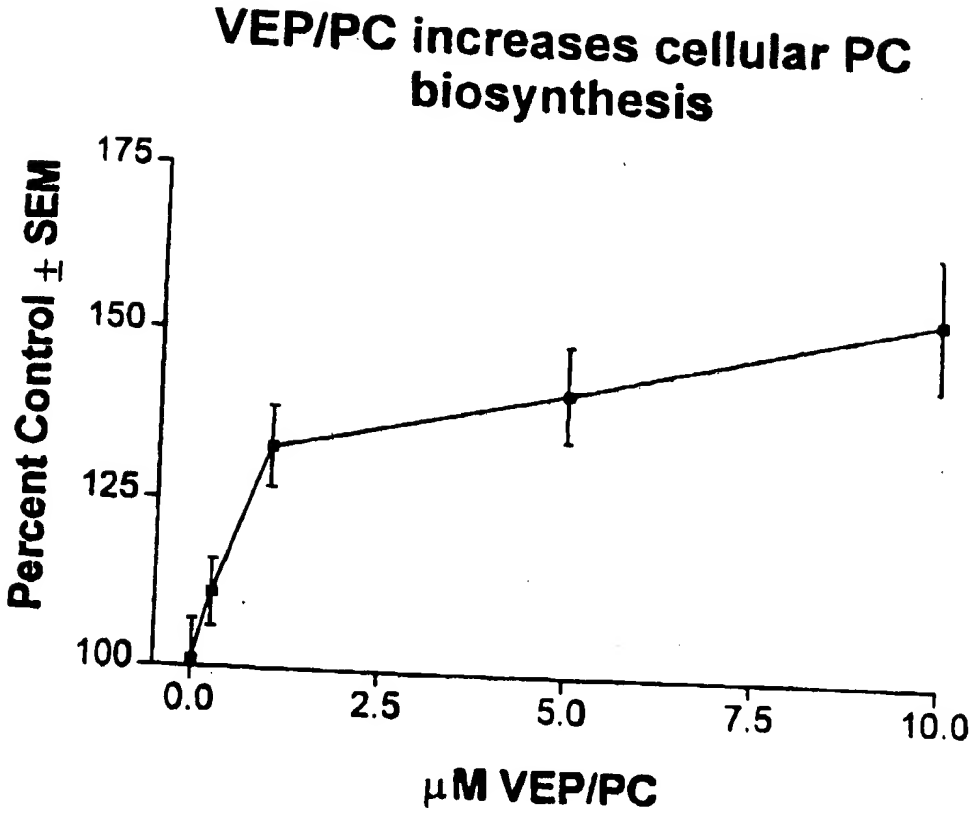


FIGURE 13



Chapter 4

NEW TRENDS IN THE PREVENTION OF HEPATOCYTE DEATH: MODIFIERS OF CALCIUM MOVEMENT AND OF MEMBRANE PHOSPHOLIPID METABOLISM

Robert G. Lamb, Jack W. Snyder, and John B. Coleman

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I. INTRODUCTION

A major obstacle to prevention of liver cell death is the failure to identify the critical cellular alterations that initiate the pathogenic scheme of cell necrosis. Resolution of this issue is complicated by the knowledge that a diversity of agents and conditions will injure and kill liver cells.[1-3] In addition, various biochemical and morphological alterations occur[1-3] when hepatocytes die, and any of these changes could be a manifestation rather than a cause of cell death. Nevertheless, there is abundant evidence suggesting that liver cell injury and death may be due in part to disturbances in calcium ion homeostasis.[2,4,5] If this conclusion is valid, then alterations in liver cell phospholipid metabolism may be a critical event in liver cell injury since: (1) the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate is a key event in the mobilization of intracellular Ca^{2+};[6] and (2) phospholipids are required to maintain the functional integrity of membranes (plasma, mitochondrial, endoplasmic reticulum) that regulate calcium homeostasis.[7] Therefore, the primary purpose of this chapter is to review data indicating that changes in the degradation and/or biosynthesis of liver cell phospholipids with subsequent disruption of membrane functions such as regulation of Ca^{2+} homeostasis may explain in part chemical- and ischemic-related hepatotoxicity.

II. ROLE OF Ca^{2+} IN LIVER CELL INJURY

Liver damage is associated with an accumulation of calcium ions.[1-5] This could mean that Ca^{2+} causes liver cell necrosis since it regulates a number of cellular functions such as secretion and metabolism.[6,8,9] However it is also possible that the accumulation of Ca^{2+} is a consequence rather than a cause of liver cell death. If calcium ions are a mediator of liver cell necrosis, then chemical- and ischemic-dependent liver cell injury must disrupt normal hepatocellular calcium homeostasis. Under normal resting conditions, the cytosolic Ca^{2+} level is low (0.2 μM) compared to the extracellular fluid (1 mM).[6] This large Ca^{2+} gradient is maintained by the combined actions of a mitochondrial Ca^{2+}-sequestering system and the Ca^{2+}-ATPases in the endoplasmic reticulum and plasma membrane.[2] Increases in cytosolic Ca^{2+} levels may be a result of a rise in the influx of extracellular Ca^{2+}, a decrease in Ca^{2+} efflux mechanisms, or some combination of these processes. It is not clear, however, whether Ca^{2+}-dependent liver cell injury is a consequence of alterations in one or all of these Ca^{2+}-homeostatic mechanisms. In fact, a major controversy exists about whether extracellular or intracellular Ca^{2+} is the critical mediator of Ca^{2+}-dependent liver cell necrosis. Farber and co-workers[6,10-12] have recently proposed a two-step sequence to explain chemical-induced liver cell injury. The first step involves an interaction with the hepatocyte to produce alterations in cellular functions (undefined) that disrupt the functional integrity of the plasma membrane. The final step is the rise in intracellular Ca^{2+} as a consequence of the influx of extracellular Ca^{2+} across the damaged plasma membrane. This hypothesis is supported by the observation that liver cell death caused by various agents (e.g., the calcium ionophore, A-23187, CCl_4, bromobenzene, lysolecithin, amphotericin B, phalloidin, etc.) is dependent on the presence of extracellular Ca^{2+}. Farber has therefore suggested that the influx of extracellular Ca^{2+} may represent a final common pathway in chemical-dependent liver cell injury and death.[6,10-12]

A. Ca^{2+} Channel Blockers

If the influx of extracellular Ca^{2+} is an important event in liver cell death, then agents that reduce this influx may prevent or reduce Ca^{2+}-mediated cell injury. This hypothesis was first supported by the observation that phenothiazines such as chlopromazine protect the liver against chemical-dependent injury.[13-15] It is postulated that phenothiazines are

protective because they inhibit the flux of Ca^{2+} across damaged membranes.[13] More recently, calcium entry blockers such as verapamil and nifedipine have also been used to prevent cellular injury caused by cytosolic calcium overload.[15-18]

Lefer and co-workers[17] have observed the beneficial actions of calcium entry blockers in the isolated perfused hypoxic cat liver. In this preparation, hypoxia is a potent stimulus for lysosomal disruption, leakage of cytoplasmic enzymes, and enhanced proteolysis. These alterations can be measured by release into the perfusate of cathepsin D, lactate dehydrogenase (LDH), and amino-nitrogen, respectively. Nontoxic (0.3 to 0.5 μg/mℓ) concentrations of nitrendipine and nifedipine infused at the onset of hypoxia resulted in significant decreases in measurable perfusate cathepsin D activity, LDH activity, and amino-nitrogen levels compared to control livers perfused with the vehicle (ethanol 0.01%). The authors concluded that inhibition of calcium influx probably was responsible for the protective effects of nifedipine and nitrendipine. However, the precise molecular mechanism for the hepatoprotection remains unclear. Lefer et al.[18] have more recently demonstrated similar hepatoprotective effects of anipamil and ronipamil (1 to 2 mg/kg) administered intravenously to cats whose livers were subsequently perfused under hypoxic conditions.

Landon et al.[15] have studied the effects of calcium-entry blockers on membrane microviscosity and calcium homeostasis in rat liver exposed to carbon tetrachloride. Polarized fluorescence of diphenylhexatriene was used as a measure of microviscosity in liver plasma membranes, microsomes, and liposomes prepared from control and CCl_4-treated rats. A progressive decline in microviscosity of these membranes was noted in the first 18 hr following CCl_4 administration. The decrease in microviscosity was correlated with significant decreases (20%) in membrane phospholipid content and followed by massive increases in calcium content of rat liver homogenates in the same 18-hr period. However, intraperitoneal administration of verapamil or nifedipine (25 mg/kg) 1 hr prior to CCl_4 treatment (2.5 mℓ/kg) partially reduced the accumulation of calcium and the degree of histological damage with only mild effects on the decreased microviscosity. The authors postulated that a critical threshold level of microviscosity may exist which is essential for adequate control of calcium entry. If the viscosity level falls too low (e.g., 12 hr after CCl_4 exposure), then calcium entry may be facilitated. Furthermore, the calcium-entry blockers may only be cytoprotective if administered early — that is, prior to a fall in membrane microviscosity below the critical threshold necessary for calcium homeostasis.

B. Role of Intracellular vs. Extracellular Ca^{2+} in Liver Cell Injury

Despite the observations that Ca^{2+} ionophores (e.g., A-21387) and Ca^{2+} channel blockers (e.g., chlorpromazine, verapamil, and nifedipine) can influence liver cell injury, there is still controversy about the role of extracellular Ca^{2+} in acute liver cell injury. Smith et al.[19] demonstrated that hepatotoxins such as CCl_4, bromobenzene, and ethyl methanesulfonate are more toxic to hepatocytes in the absence rather than the presence of extracellular Ca^{2+}. Reed and colleagues[20-23] have also noted that various agents are more toxic to liver cells incubated without extracellular Ca^{2+} as compared to cells incubated with Ca^{2+}. In fact, these investigators suggest that extracellular Ca^{2+} protects liver cells against injury.[21,23] The protective effect of extracellular calcium may be due in part to the decrease this ion produces in cellular functions that are associated with cell injury such as lipid peroxidation, oxidative stress, and glutathione depletion. These cellular processes are increased in a medium containing Ca^{2+} as compared to cells incubated in a calcium-free medium.[21,23] Fariss et al.[23] also observed that hepatocytes incubated in a Ca^{2+}-supplemented medium had higher levels of the antioxidant vitamin E than cells maintained in a Ca^{2+}-free medium. Therefore, extracellular calcium may be protective because this ion maintains the level of cellular components (glutathione and vitamin E) that may prevent chemical-mediated liver cell injury.[21,23]

There is obviously a contradiction concerning the role of extracellular Ca^{2+} between Farber's[4,10-12] results and those of Reed[20-23] and Orrenius.[5,19] Farriss et al.[20] have suggested that this discrepancy may be a result of the presence or absence of vitamin E in the liver cell incubation medium. Apparently, the medium (Williams medium E) used in Farber's studies contained vitamin E whereas that used by the co-workers of Reed and Orrenius did not.[23] Extracellular Ca^{2+} is toxic to liver cells when vitamin E is present and protective when vitamin E is absent.[23] Therefore, the liver cell toxicity observed by Farber and co-workers[4,10-12] may be a result of vitamin E and not Ca^{2+} per se.[23] This conclusion is supported by the observation that vitamin E prevents chemical-mediated liver cell injury in the absence of Ca^{2+}[23] and that marked increases (eightfold) in intracellular Ca^{2+} levels are not necessarily toxic.[20,22]

Chemical-mediated alterations in Ca^{2+} homeostasis may also be a result of changes in intracellular Ca^{2+}-homeostatic mechanisms. Bellomo et al.[24] have developed techniques to determine the influence of agents on the Ca^{2+} content of the mitochondrial and/or extramitochondrial compartments. Sequential addition of the protonophore uncoupler carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone and the Ca^{2+} ionophore A-23187 causes a release of Ca^{2+} from the mitochondrial and extramitochondrial compartments, respectively. The independent release of Ca^{2+} from each compartment is measured by changes in the absorbance of the metallochromic indicator, Arsenazo III, as it binds the released Ca^{2+}. Orrenius and co-workers[5,24-27] have used this technique extensively to study the effects of chemicals on liver cell calcium homeostasis.

Their observations suggest that mitochondrial Ca^{2+} content is controlled by the redox state of pyridinine nucleotides (NADPH)[5,24] whereas that of the plasma membrane[26] and endoplasmic reticulum[27] is sensitive to the redox state of thiol groups. Therefore, chemicals that selectively oxidize NADPH or glutathione will decrease the Ca^{2+} content of mitochondrial and extramitochondrial pools, respectively.[5,24] Orrenius and associates[5,24-28] have concluded that various hepatotoxins (bromobenzene, *t*-butyl hydroperoxide, acetaminophen) may disrupt Ca^{2+} homeostasis by decreasing the level of glutathione that is necessary to maintain the functional status of Ca^{2+}-binding proteins (calmodulin) and the endoplasmic reticulum Ca^{2+} pump.[25] The protective role of thiols such as glutathione is supported by the observation that dithiothreitol (a thiol reagent) prevents extramitochondrial Ca^{2+} loss and cell surface blebbing (early toxic event) in hepatocytes incubated with *t*-butyl hydroperoxide.[24] A similar conclusion was reached by Jewel et al.,[25] using different toxic agents. Other investigators have also concluded that reductions in the capacity of the endoplasmic reticulum to sequester Ca^{2+} may be a critical event in chemical-mediated liver cell injury.[29-32]

III. THE ROLE OF PHOSPHOLIPID METABOLISM IN LIVER CELL INJURY

Singer and Nicholson[33] have described a dynamic equilibrium between phospholipids and proteins in their fluid mosaic model of mammalian membrane structure. In this membrane model, a phospholipid bilayer contains integral proteins that function as transporters, enzymes, and receptors. Most likely, the functional integrity of these membrane proteins is dependent on the membrane's ability to maintain the bilayer's normal phospholipid composition and content. This concept is supported by the observation that enzyme activity, ion transport, and receptor function are altered in membranes when their phospholipid content is changed.[7,34-36] Therefore, alterations in membrane function that occur when liver cells are injured[2] may be a result of changes in membrane phospholipid content.

Reduction in hepatocellular phospholipid content have been observed during chemical-[15,37-40] and ischemic-dependent[41,42] liver cell injury. However, it is not apparent whether the decrease in membrane phospholipid is a cause or a result of membrane injury. Also, the

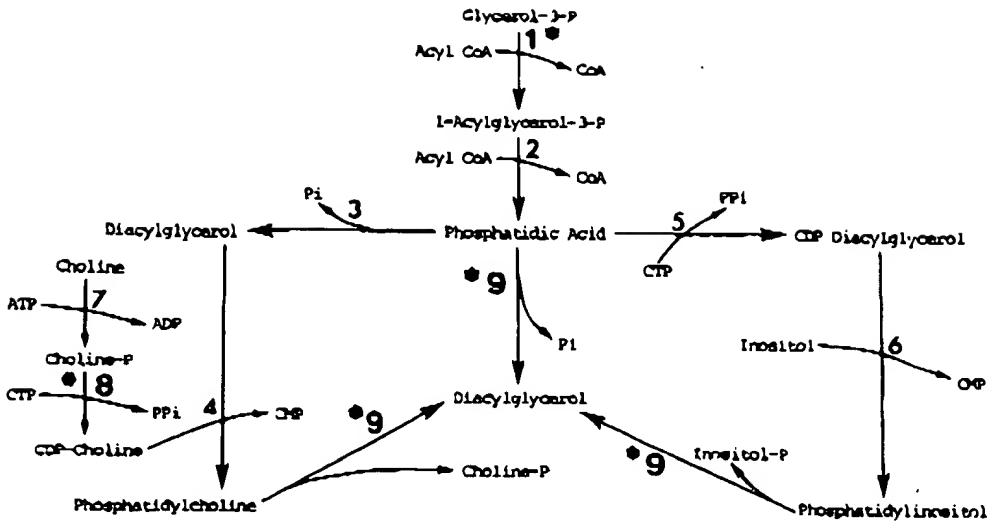

FIGURE 1. An outline of the biochemical reactions involved in the formation and degradation of hepatocellular phospholipids. Labeled glycerol-3-P is sequentially esterified by sn-glycerol-3-P acyltransferase (Reaction 1) and 1-acylglycerol-3-P acyltransferase (Reaction 2) to form phosphatidic acid. Phosphatidic acid is either dephosphorylated (Reaction 3) to diacylglycerol or converted to CDP diacylglycerol (Reaction 5). The CDP choline generated by phosphocholine cytidylyltransferase (Reaction 8) is utilized by cholinephosphotransferase (Reaction 4) to convert diacylglycerol to phosphatidylcholine. CDP diacylglycerol is incorporated into phosphatidylinositol by phosphatidylinositol synthetase (Reaction 6). Phosphatidylinositol, phosphatidylcholine, and phosphatidic acid are hydrolyzed by phospholipase C (Reaction 9) to form diacylglycerol with the release of inositol-P, choline-P, and P_i, respectively.

mechanism by which membrane phospholipid content is reduced is not clear. Obviously, the concentration of membrane phospholipids is influenced by changes in phospholipid production, degradation, or some combination of these processes. Unfortunately, there is a limited knowledge of how liver cells regulate phospholipid metabolism in order to maintain the normal phospholipid content of membranes. Recent studies from this laboratory, however, suggest that enzyme-mediated changes in the formation and degradation of hepatocellular phospholipids may be a key event in chemical-dependent liver cell injury.[37-40] The reactions catalyzed by these phospholipid-metabolizing enzymes are outlined in Figure 1.

A. Ischemic- and Chemical-Dependent Alterations in Phospholipid Metabolism

Farber and co-workers[41,42] have demonstrated that hepatic ischemia reduces the level of hepatic phospholipids. In fact, 25 to 35% of the total hepatic phospholipid content is reduced after 3 hr of ischemia. The degradation of membrane phospholipids may be an important event in ischemic-dependent liver cell injury since Chien et al.[41] have observed that: (1) the time course of phospholipid depletion parallels the onset of irreversible liver injury; (2) the degree of membrane dysfunction is directly proportional to the reduction in membrane phospholipid content; (3) membrane dysfunction is reversed by replacing degraded membrane phospholipids; and (4) agents such as chlorpromazine that inhibit the ischemic-dependent degradation of membrane phospholipids also decrease the ischemic-associated dysfunction of membranes and hepatic injury. More recently, Finkelstein et al.[42] have observed that ischemia also reduces the capacity of the liver to esterify phospholipids. Therefore, ischemia reduces the phospholipid content of hepatic membranes by increasing and decreasing phospholipid degradation and biosynthesis, respectively. Results shown in Figure 2 support this conclusion and suggest that hepatotoxins, such as carbon tetrachloride, may produce alterations in hepatic phospholipid metabolism in vivo that are similar to those produced by

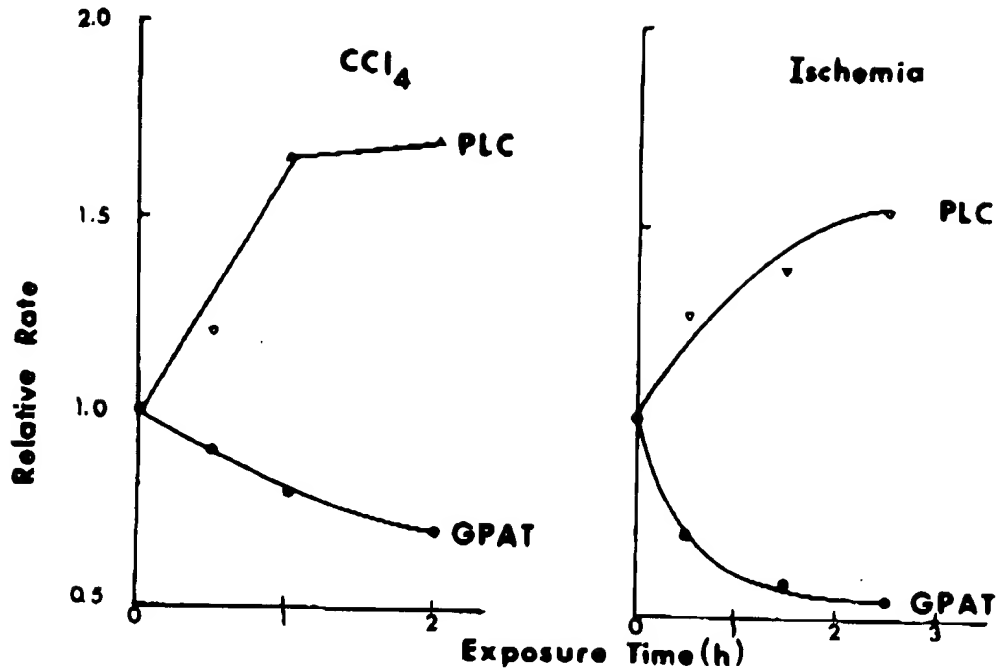

FIGURE 2. The time course of CCl_4- and ischemic-dependent effects on the activity of rat liver phospholipase C (PLC) and glycerol-3-phosphate acyltransferase (GPAT). Relative rate is expressed as a function of control enzyme activity (0 exposure time) which is equal to 1.0. Rats were exposed to a single i.p. dose (1.0 ml/kg body weight) of corn oil/CCl_4 (1/1 v/v). Each point represents the mean of data obtained from five animals.

ischemia. Both ischemia and carbon tetrachloride exposure cause time-dependent increases and decreases in hepatic phospholipase C and sn-glycerol-3-P acyltransferase activity, respectively. These alterations in enzyme activity would decrease membrane phospholipid content since phospholipase C influences the degradation and sn-glycerol-3-P acyltransferase the biosynthesis of liver cell phospholipids (Figure 1).

B. Isolated Hepatocyte Studies

It is not apparent how ischemia and chemical exposure alter hepatic phospholipid metabolism in the intact animal. Therefore, primary cultures of adult rat hepatocytes have been utilized to study the influence of anoxia[43] and hepatotoxins[38-40] on liver cell phospholipid metabolism in vitro. Farber and Young[43] demonstrated that anoxia rapidly accelerated the degradation of liver cell membrane phospholipids such as phosphatidylcholine and phosphatidylethanolamine. The anoxia-dependent increase in phospholipid degradation is most likely a result of a rise in hepatocellular phospholipase C activity since there is a marked increase in hydrophilic degradation products such as phosphorylethanolamine and phosphorylcholine (Figure 1). The rapid rise (1 to 2 hr) in the phospholipase C-mediated degradation of membrane phospholipids is associated with membrane perturbations since glucose-6-phosphatase is significantly depressed (50%) within 3.5 hr. Apparently, anoxia influences liver cell phospholipid degradation in a manner that is similar to that produced by ischemia.[41] It is intriguing to note that liver cells incubated with hepatotoxins, such as bromobenzene,[38-40] carbon tetrachloride,[38-40] and acetaldehyde,[44] also display an increase in phospholipase C-dependent degradation of membrane phospholipids. Perhaps, ischemic- and chemical-mediated liver cell injury might be due in part to a rise in the phospholipase C-dependent degradation of membrane phospholipids.

schemia

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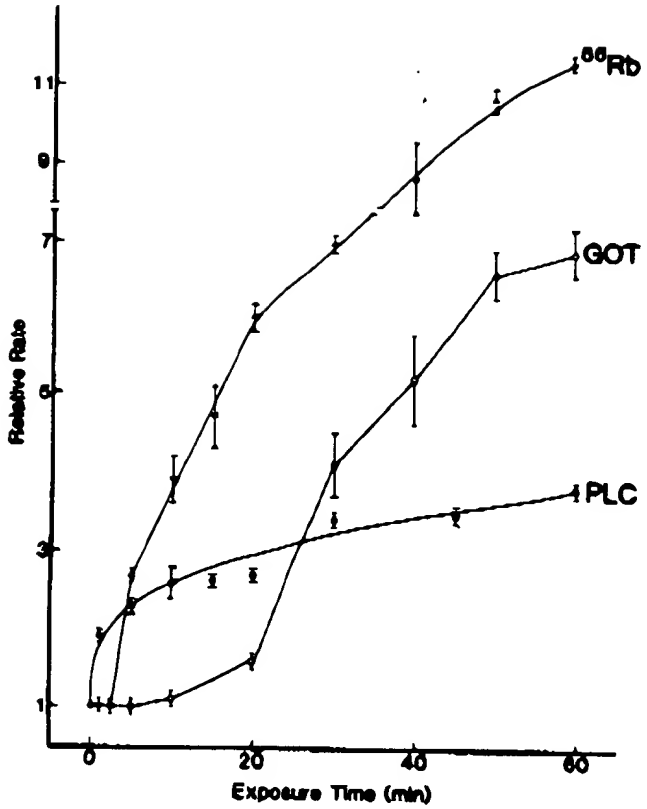

FIGURE 3. The time course of 5 m*M* bromobenzene effects on cultured hepatocyte phospholipase C (PLC) activity, ^{86}Rb loss, and glutamic-oxaloacetic transaminase (GOT) release. Each experimental value is the mean relative rate ± SEM of two to three determinations. See legend of Figure 2 for a definition of relative rate. (From Lamb, R. G., McCue, S. B., Taylor, D. R., and McGuffin, M. A., *J. Pharmacol. Appl. Toxicol.*, 75, 515, 1984. With permission.

It is not clear whether chemical-dependent changes in membrane phospholipid metabol are a cause or consequence of liver cell injury. Therefore, this laboratory has develo techniques to monitor the time course of hepatotoxin-mediated changes in the functi and structural integrity of cultured hepatocytes. When liver cells are incubated with 5 bromobenzene, there is a rapid (1 min), two- to threefold increase in phospholipase C acti that is maintained throughout the incubation period (Figure 3). The bromobenzene-depen rise in phospholipase C activity precedes the formation of cell surface "blebs" and release of intracellular ions such as ^{86}Rb. These early, hepatotoxin-mediated changes in function are probably associated with a reversible phase of cell injury.[2] However, the rel of cytosolic enzymes (e.g., GOT) following 20 min of bromobenzene exposure (Figure is most likely a result of severe, irreversible membrane damage. Perhaps the bromobenz initiated, exposure time-dependent rise in the severity of membrane damage represen progressive increase in the phospholipase C-mediated degradation of membrane phosp pids. The observation that the activation of phospholipase C precedes the bromobenz dependent disruptions in membrane function (Figure 3) suggests that the rise in the pl pholipase C-mediated degradation of membrane phospholipids is more likely a cause ra than a result of liver cell injury.

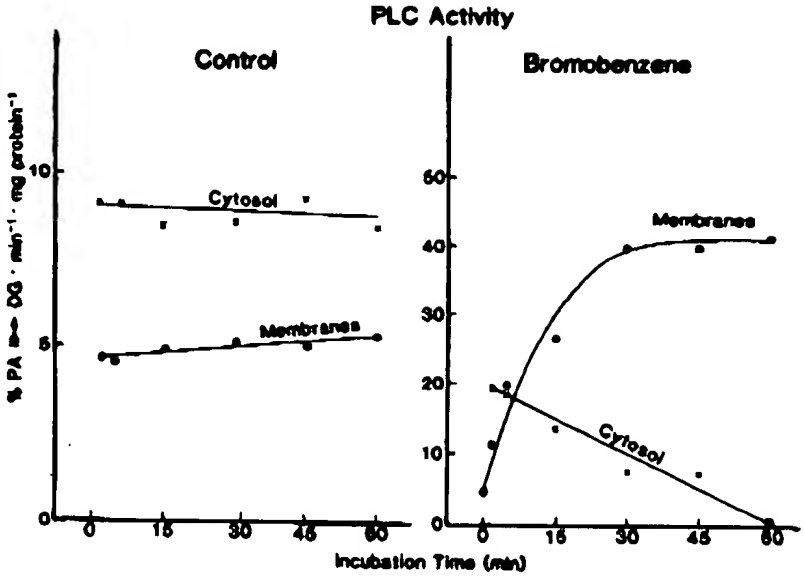

FIGURE 4. The influence of incubating (0 to 60 min) cultured hepatocytes with DMSO (control) or 5 m*M* bromobenzene on the cellular distribution of phospholipase C (PLC). Phospholipase C activity was measured as the percent of labeled, membrane-bound phosphatidic acid (PA) converted to diacylglycerol (DG) · min^{-1} · mg $protein^{-1}$. Each experimental point represents the mean of two to three separate determinations.

The rapid hepatotoxin-mediated increase in phospholipase C activity noted above is not influenced by inhibitors of protein synthesis such as cycloheximide (unpublished observation). Therefore, the rise in phospholipase C activity is probably a result of enzyme activation rather than new protein synthesis. This conclusion is supported by the data in Figure 4 which suggest that liver cells incubated with 5 m*M* bromobenzene display rapid alterations in the distribution of cytosolic- and membrane-associated phospholipase C. These preliminary studies suggest that liver cell phospholipase C may be rapidly activated by the translocation of this enzyme from the cytosol to membranes. The rise in membrane-associated phospholipase C might be the mechanism by which ischemia[41,42] and hepatotoxins[15,37-40] produce rapid increases in the degradation of membrane phospholipids.

It seems unlikely that a cell would have a rapidly activated process that leads to cell death. Most likely, the activation of phospholipase C is a mechanism by which the cell normally regulates the turnover of membrane phospholipids. In fact, it is well established that the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate is a key step in cellular signal transduction.[6,43] Nevertheless, hepatotoxins such as carbon tetrachloride and bromobenzene activate a nonspecific phospholipase C-like activity that degrades various membrane phospholipids including phosphatidylinositol.[38] Perhaps, hepatotoxins interact with phospholipids in the membrane bilayer, and the cell tries to remove these abnormal membrane phospholipids by binding cytosolic phospholipase C (Figure 4). The phospholipase C-mediated degradation of membrane phospholipids would be irreversible unless the capacity of the membrane to make new phospholipids was increased. Apparently, when membrane phospholipids, such as phosphatidylcholine are degraded by exogenous phospholipase C (*Clostridium perfringens*) the capacity of the cell to make phosphatidylcholine is correspondingly increased.[46,47] The rise in phosphatidylcholine biosynthesis is a result of the translocation of cytosolic phosphocholine cytidylyltransferase to phosphatidylcholine-depleted membranes.[47] The results shown in Figure 5 demonstrate that cultured

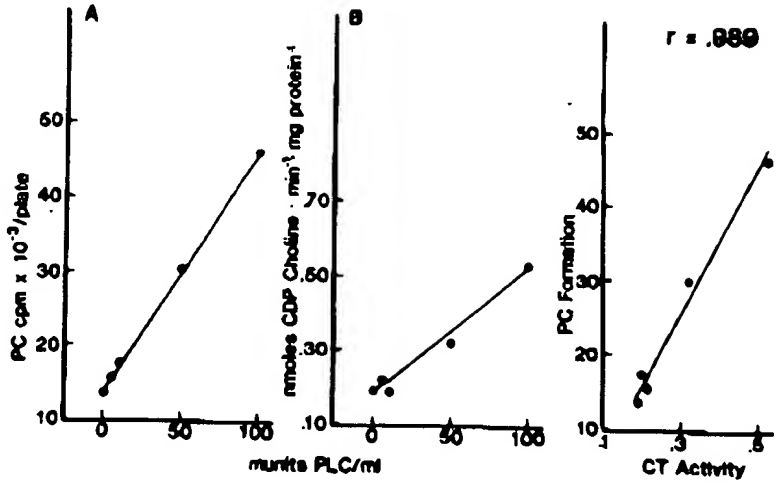

FIGURE 5. The effect of incubating (1 hr) cultured hepatocytes with various concentrations (0 to 100 m units) of exogenous phospholipase C *(C. perfringens)* on the incorporation of labeled choline into phosphatidylcholine (A) and phosphocholine cytidylyltransferase activity (B). Phospholipase C-dependent changes in PC formation (A) and phosphocholine cytidylyltransferase activity (B) are related since these parameters exhibit an excellent linear correlation ($r = 0.989$). Each point represents the mean of two to three individual experimental determinations.

hepatocytes incubated 60 min with various concentrations of exogenous phospholipase C also display dose-dependent increases in the incorporation of labeled choline into phosphatidylcholine (Figure 5A) that correlate well ($r = 0.989$) with an increase in phosphocholine cytidylyltransferase activity (Figure 5B). The rise in phosphocholine cytidylyltransferase activity may represent the attempt of the cells to make new membrane phosphatidylcholine, since this enzyme regulates liver cell phosphatidylcholine biosynthesis and content.[48] Results shown in Figure 6 suggest that cultured hepatocytes incubated with 5 m*M* bromobenzene for various times also display a rise in membrane-associated phosphocholine cytidylyltransferase activity that is a result of the translocation of cytosolic phosphocholine cytidylyltransferase. These latter results (Figure 6) support the concept that hepatotoxin-mediated alterations in liver cell phospholipid metabolism may be a result of a rise in membrane-associated phospholipase C activity.

These studies suggest that there may be a steady-state relationship between membrane phospholipid biosynthesis and degradation. Most likely, the cytosol is a reservoir of regulatory enzymes that are bound or released by membranes as needed to regulate the formation (cytidylyltransferase) or degradation (phospholipase C) of membrane phosphatidylcholines. Perhaps, the phospholipase C-mediated degradation of membrane phospholipids is reversible as long as the capacity of the membrane to make phospholipids is correspondingly increased. However, the membrane may be irreversibly injured when its phospholipids are degraded more rapidly than they are formed. This concept is supported by the results displayed in Figure 7. Cultured hepatocytes incubated with variable levels of exogenous phospholipase C *(C. perfringens)* display incubation time-dependent increases and decreases in phosphocholine cytidylyltransferase (CT) and sn-glycerol-3-P acyltransferase (GPAT) activity. These phospholipase C-mediated changes in enzyme activity should produce corresponding alterations in membrane phospholipid biosynthesis since both enzymes catalyze key reactions in hepatic phospholipid biosynthesis (Figure 1).[49] Therefore, the initial phospholipase C-dependent rise in enzyme activity (Figure 7), may represent the attempt of the cell to repair the phospholipid-depleted membranes (reversible); however, irreversible injury may occur

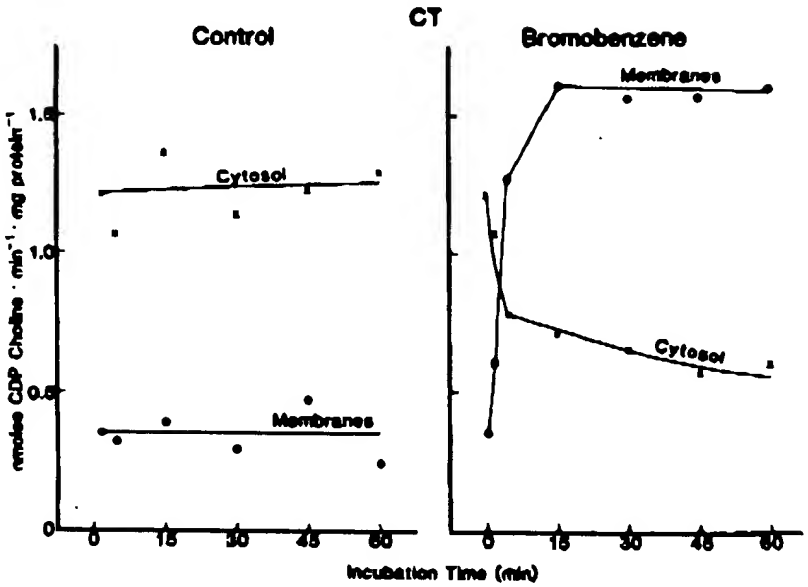

FIGURE 6. The effect of incubating (0 to 60 min) cultured hepatocytes with DMSO (control) or 5 m*M* bromobenzene on the cellular distribution of phosphocholine cytidylyltransferase (CT). Cytidylyltransferase activity was expressed as the nmoles of CDP choline formed · min^{-1} · mg $protein^{-1}$. Each point is the mean of two to three separate experimental values.

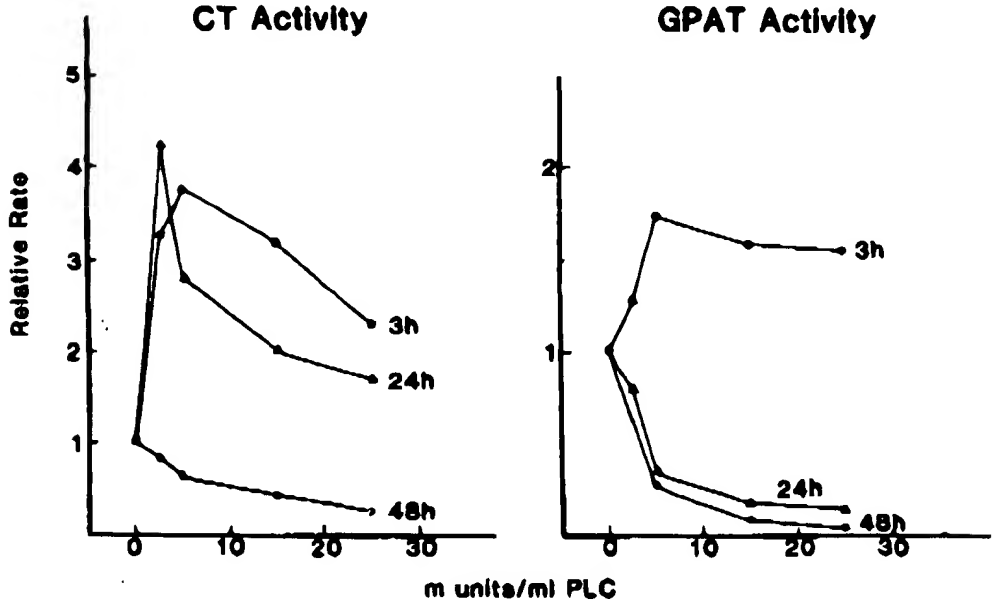

FIGURE 7. Alterations in phosphocholine cytidylyltransferase (CT) and glycerol-3-P acyltransferase activity that occur when cultured hepatocytes are incubated (3, 24, and 48 hr) with various levels of exogenous phospholipase C (*C. perfringens*). Each value is the mean of two to three individual values. Relative rate is defined in the legend of Figure 2.

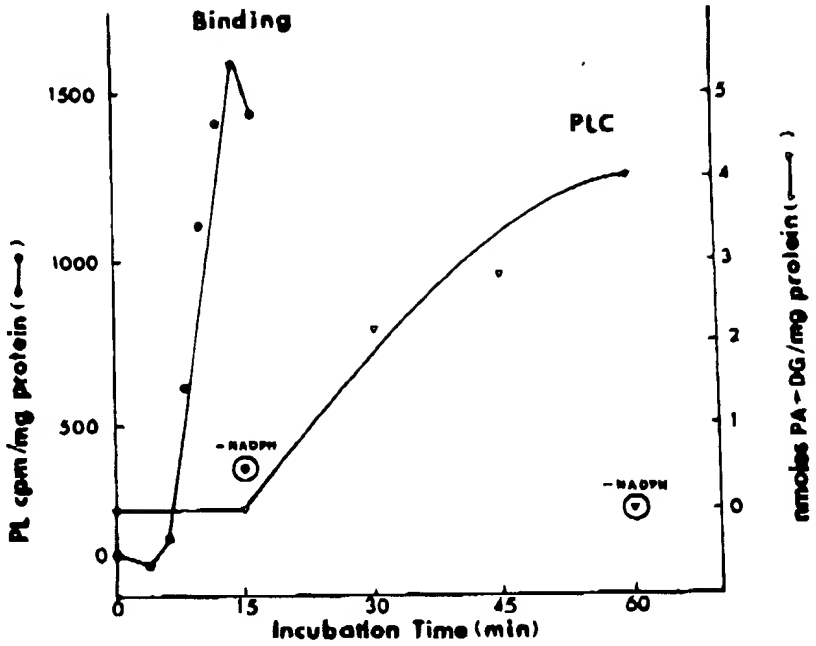

FIGURE 8. The relationship between the covalent binding of NADPH-dependent CCl_4 metabolites and the CCl_4-(0.5 m*M*) mediated rise in cultured hepatocyte phospholipase C (PLC) activity. The influence of deleting NADPH on binding and phospholipase C activity is shown at 15 and 60 min (NADPH), respectively. Each point is the mean of three separate determinations.

when the activity of these enzymes is significantly reduced (Figure 7). Perhaps, the phospholipase C-mediated reduction in phosphocholine cytidylyltransferase and sn-glycerol-3-P acyltransferase activity is a result of membrane phospholipid depletion, since both enzymes are phospholipid-dependent.[48,50]

Previous work has established that chemical-dependent liver cell injury may be a result of changes in peroxidation and alkylation.[51] However, the relationship between these processes and liver cell injury is still unresolved. This relationship may exist because peroxidation and alkylation alter the structure of membrane phospholipids. These abnormal phospholipids may be the signal that causes the translocation of cytosolic phospholipase C to membranes. The membrane-associated phospholipase C will decrease membrane phosphatidycholine content by degrading the abnormal phosphatidylcholines. Phosphatidylcholine-deficient membranes will increase their capacity to make phosphatidylcholines by binding cytosolic phosphocholine cytidylyltransferase. Hence, membranes bind or release phospholipase C and phosphocholine cytidylyltransferase as needed to maintain the membrane phosphatidylcholine content. This concept is supported by the data in Figure 8. The binding of labeled, CCl_4 metabolites to membrane phospholipids precedes CCl_4-dependent rise in phospholipase C activity. Also there is a good correlation in vitro between phospholipid-bound CCl_4 metabolites and the activation of phospholipase C. Similar results have also been obtained in intact animal studies (unpublished). Sipes and Gandolfi[32] also concluded that the binding of reactive metabolites to phospholipids correlated resonably well with the hepatotoxicity of various halogenated hydrocarbons in vivo. Therefore, membrane phospholipids may be an important target molecule of chemically reactive metabolites.

Alterations in the phospholipase C-mediated degradation of membrane phospholipids may also explain in part the changes in calcium homeostasis that are associated with chemical-

and ischemic-dependent liver cell injury.[2,4,5] For example, it is well established that the phospholipase C-dependent hydrolysis of a phosphatidylinositol 4,5-biphosphate is associated with the mobilization of intracellular Ca^{2+}.[6,45] In addition, the functional integrity of membrane Ca^{2+} pumps is markedly disrupted when membranes are incubated with exogenous phospholipase C.[53,54]

It would be appropriate at this stage to have more discussion on modifiers of phospholipid metabolism and their influence on liver cell death. However, this is still an unresolved area of research. Unfortunately, there is a current lack of agents that are specific inhibitors and/or activators of hepatocellular phospholipid metabolism.

ACKNOWLEDGMENTS

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An *In Vitro* Model of Ethanol-dependent Liver Cell Injury

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Primary cultures of adult rat hepatocytes were incubated (6 to 96 hr) with 50 to 150 mmol/L ethanol, 0.5 mmol/L linoleate, 0.5 mmol/L palmitate, 0.5 mmol/L 4-methylpyrazole, 0 to 25 μmol/L vitamin E phosphate or selected combinations of these agents. Agent-dependent changes in liver cell viability (AST release and reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and function (phospholipid peroxidation, hydrolysis, biosynthesis and triacylglycerol biosynthesis) were determined. The influence of ethanol on liver cell function and viability was dose and incubation time dependent. Short periods (24 hr or less) of exposure to 100 mmol/L ethanol increased liver cell triacylglycerol biosynthesis and phospholipid hydrolysis, peroxidation and biosynthesis without altering cell viability. However, longer periods (72 hr or more) of exposure to 100 or 150 mmol/L ethanol resulted in significant reductions (30% to 50%) in cell viability, function and phosphatidylcholine biosynthesis and content. The ethanol-dependent decreases in cell function and viability were potentiated by linoleate and reduced by vitamin E phosphate, palmitate and 4-methylpyrazole. These results suggest that ethanol-induced liver cell injury *in vitro* is not a result of ethanol *per se*, but factors such as acetaldehyde or oxyradicals produced as a consequence of ethanol metabolism. Therefore the incubation of cultured hepatocytes with ethanol may be an appropriate model *in vitro* for determining the mechanisms by which ethanol intake disrupts liver cell function *in vivo*. (HEPATOLOGY 1994;19:174-182.)

Alcohol-dependent liver disease (ALD) is the most common form of liver disease in the western world. ALD is also the major cause of chronic medical illness and death associated with alcohol abuse (1). Despite the frequency of ethanol-dependent liver injury, understanding of the mechanisms by which ethanol ingestion results in ALD is limited. This lack of knowledge is primarily a result of the inability of investigators to produce ALD in rodents that voluntarily ingest liquid ethanol diets. Apparently, rodents do not ingest enough ethanol in the liquid diets because the animals develop a fatty liver but no necrosis. More recently, however, investigators have overcome the ethanol administration problem by continuous intragastric infusion of ethanol to rats (2-6). Under these conditions, blood ethanol levels were cyclic, with a peak magnitude ranging from 300 to 450 mg/dl or 65 to 100 mmol/L. After 30 days of ethanol infusion, the livers had fat infiltration and zonal necrosis (2, 3). The ethanol-induced liver injury was potentiated by unsaturated fatty acids such as linoleate (5, 6) and reduced by saturated fatty acids (5, 6). These studies are important because they demonstrate the following: (a) the rat may be an appropriate model of ALD; (b) high blood ethanol levels are required to produce ALD; and (c) unsaturated (linoleate) but not saturated fatty acids markedly potentiate ethanol-dependent liver cell injury *in vivo*. These results also suggest that primary cultures of adult rat hepatocytes may be an appropriate *in vitro* model of ethanol-induced liver cell injury, if primary cultures incubated with ethanol mimic the liver cell's response to ethanol and fatty acids *in vivo*.

The primary objective of this paper is to demonstrate that primary cultures of adult rat hepatocytes incubated (6 to 96 hr) with various concentrations (50 to 150 mmol/L) of ethanol, fatty acids (linoleate and palmitate) or both display ethanol- and fatty acid–dependent alterations in cell function and viability *in vitro* that mimic the effects of ethanol and fatty acid exposure in the intact animal, such as fat accumulation and necrosis. These studies also suggest that factors generated during the metabolism of ethanol, such as acetaldehyde, oxyradicals or both, may result in cell injury because ethanol-induced liver cell injury is blocked by 4-methylpyrazole (4MP) and vitamin E phosphate (VEP). Therefore, primary cultured hepatocytes incubated with ethanol may be an appropriate model *in vitro* for determining the mechanisms by which ethanol induces liver cell injury *in vivo*.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Dominion Laboratories, Dublin, VA) weighing 300 to 350 gm were kept on a

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12-hr light-dark cycle and fed a standard diet and water *ad libitum*? Animals were handled in compliance with the Insti-tutional Animal Care and Use Committee at Virginia Commonwealth University.

Reagents. Cytidine 5'-diphospho[Me-^{14}C]choline, [^{3}H]choline and [1,3-^{14}C]glycerol were purchased from Du Pont–New England Nuclear (Boston, MA). Collagen (type III), EDTA, [5,5'-dithiobis-(2-nitrobenzoic acid)] (DTNB), Trizma-7.5, dexamethasone, testosterone benzoate, vitamin E, VEP, β-estradiol benzoate, aminolevulinate, sodium selenate, linoleate, palmitate, oleate, $MgCl_2$, glucose and culture amino acids were obtained from Sigma Chemical Co., St. Louis, MO. DMSO was secured from Aldrich Chemical Co., Milwaukee, WI. Culture plates (35-mm contour) were obtained from Lux Scientific Corp., Newbury Park, CA. Gentamycin, penicillin, ethanol and insulin were obtained from the Medical College of Virginia Hospital Pharmacy. Minimum essential vitamin mix and glutamine were purchased from Microbiological Associates, Walkersville, MD. All culture media (Waymouth 752/1) were prepared in our laboratory and sterilized by membrane filtration (0.45-μm pore size; Millipore Corp., Bedford, MA). Each day, fresh medium was supplemented (0.1 ml DMSO/100 ml) with a 0.1-ml aliquot of DMSO containing 79 μg testosterone benzoate, 75 μg β-estradiol benzoate, 79 μg dexamethasone, 500 μg vitamin E, 500 μg oleate and 500 μg linoleate. All lipid standards were purchased from Applied Science Labs Inc., State College, PA. BIO-SIL A for column chromatography and Silica Gel 60 thin-layer chromatography plates were obtained from Bio-Rad Laboratories (Melville, NY) and Whatman, Inc. (Clifton, NJ), respectively.

Cultured Hepatocytes. Hepatocytes were isolated (7) from the livers of rats (300 to 350 gm) pretreated with a 50 mg/kg intraperitoneal dose of phenobarbital 3 days before the liver perfusion. During this 3-day period, the animals were also given *ad libitum* water containing 0.1% sodium phenobarbital. Hepatocytes were isolated and sedimented by centrifugation (70 *g* for 3 min), washed and subjected to an isodensity Percoll (20%) centrifugation to remove nonviable cells as previously described (8). Cells were washed three times with culture medium, and aliquots (1×10^6 cells) were placed in 35-mm plastic culture dishes (precoated with type III collagen) containing 1.25 ml of modified Waymouth 752/1 medium (8, 9). Ten microliters of rat plasma was added to each plate, and 1 to 2 hr later 0.010 mmol/L VEP was added as a dioleoyl phosphatidylcholine (PC) liposome. Liposomes were prepared by sonicating (10 to 15 min) mixtures (1:4) of VEP and PC in water at 37° C. VEP's cytoprotective effects may be a result of its ability to inhibit lipid peroxidation and to stimulate membrane repair systems such as PC biosynthesis (10). Aliquots (0.01 ml) of DMSO and water or DMSO and water containing variable amounts of fatty acids and ethanol were also added to the medium of 2-hr monolayers (covered with cups), and cell functions were assessed at 24-hr intervals. Old medium was removed and new medium and agents were added at 24-hr intervals.

Isotope Incorporation. After the appropriate agent-incubation period, a 0.02-ml aliquot of either [^{3}H]choline or [^{14}C]glycerol was added to the media to give a final concentration of 0.1 mmol/L (0.1 μCi). Incubations were stopped after 90 min by the addition of 2 ml of methanol. Cells were removed from the culture dishes by scraping with a Teflon-coated spatula and placed in glass test tubes. Two milliliters of chloroform and 0.5 ml of 0.08N HCl were added to each tube. Lipids were extracted and washed by a modified Bligh and Dyer technique (11). Lipids were separated into neutral and phospholipid fractions by techniques previously described (12), and labeled lipids were counted in a Beckman beta counter (Beckman Bioanalytical Systems Group, Fullerton, CA). The content of PC was determined with the Bartlett (13) procedure for measuring inorganic phosphate. The control rate (picomoles per minute per milligram protein) of glycerol and choline incorporation varied significantly (twofold) from preparation to preparation. As a result, most data are expressed as a percentage of control so that data from several preparations can be combined. Control values are given in the appropriate figure or table legend. Each data point represents the mean ± S.E.M. of 3 to 9 separate determinations. The probable significant level (p) between each experimental and control group was determined by Student's *t* test.

Cell Viability. Agent-dependent changes in cell viability were measured by several techniques (14, 15). Determination of the proportion of cells that excluded 0.2% trypan blue was routinely used to assess cell viability. Cell viability was also assessed by determining the level of cytosolic AST in the cell medium, as described by the protocol in the Sigma GO-Transaminase Kit no. 505 (Sigma Chemical Co.). Viability was also determined by colorimetrically measuring the amount of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduced by viable cells (14-17). Rates of PC biosynthesis (see Isotope Incorporation) were also used to assess cell viability (14). Each of these techniques is appropriate for determining agent-induced alterations in cell viability; however, MTT reduction (15) and lipid biosynthesis (14) were the most sensitive measures of cell viability.

Enzyme Assays. Medium was aspirated from the plates, and cells were scraped into 2 ml of liver buffer containing 0.225 mol/L sucrose, 0.05 mol/L Tris-HCl (pH 7.5) and 2.5 mmol/L EDTA. Cells were sedimented by centrifugation at 700 *g* for 2 min, buffer was removed by aspiration and the cells were resuspended in 2 ml of sucrose/Tris/EDTA buffer. Cells were homogenized for 15 sec at a setting of 5 on a Technicon Homogenizer (Technicon Instrumentation, Miles Diagnostics, Inc., Carytown, NY). This cell homogenate was used as an enzyme source to measure phosphocholine cytidylyltransferase (PCT) and phospholipase C (PLC) activity. PLC activity was measured in membrane fractions by techniques previously outlined (18). PCT activity was determined by a modification of the procedure described by Weinhold, Rounsifer and Feldman (19) with PC-oleic acid vesicles. Alcohol dehydrogenase was measured in monolayer homogenates by techniques outlined by Mezey, Potter and Rhodes (20). Protein was determined as described by Lowry et al. (21) with BSA as a standard.

Lipid Peroxidation. One milliliter of PBS containing 137 mmol/L NaCl, 2.68 mmol/L KCl, 8 mmol/L Na_2HPO_4 and 2.5 mmol/L EDTA (pH 7.0) was added to each culture plate. Cells were removed by scraping with a Teflon-coated spatula and placed in glass test tubes. One milliliter of 10% trichloroacetate was added, and the mixture was vortexed and centrifuged for 3 min. Two 1.2-ml aliquots of supernatant were removed and placed in separate glass test tubes. Malondialdehyde (thiobarbituric acid–reacting substances) was measured as previously described (22).

Glutathione Determination. Liver cell glutathione content was determined as described by Sedlak and Lindsay (23). Hepatocyte monolayers were rinsed with liver buffer (see Enzyme Assays), 2 ml of ice-cold 20 mmol/L disodium EDTA was added and cells were removed by scraping and placed in glass test tubes. Cells were homogenized, and proteins were precipitated with 10% trichloroacetic acid. The absorbance of clear supernatant in the presence of DTNB was determined at 412 nm (23).

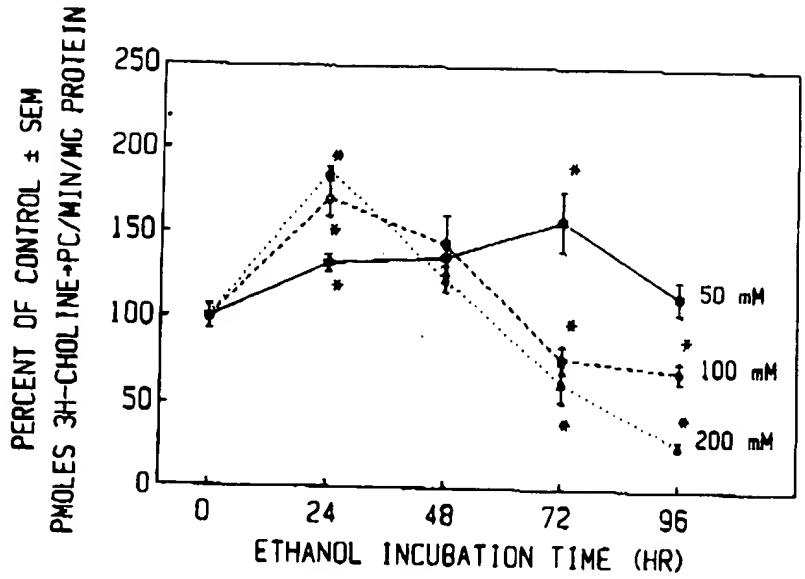

FIG. 1. Alterations in the incorporation of [^{3}H]choline into PC of hepatocyte monolayers incubated (0 to 96 hr) with various doses of ethanol (50 to 200 mmol/L). Each determination represents the mean ± S.E.M. of 3 to 9 experimental values. Control values are 196 pmol of [^{3}H]choline incorporated into PC per minute per milligram protein ± 12. *Significantly different from control ($p \leq 0.01$).

RESULTS

The dose-dependent (50 to 150 mmol/L) and exposure time–dependent (24 to 96 hr) effects of ethanol on [^{3}H]choline incorporation into PC by hepatocyte monolayers are shown in Figure 1. At 24 hr of incubation, all doses of ethanol significantly increase PC biosynthesis. After longer periods (72 hr or more) of exposure, all doses except 50 mmol/L decrease (30% to 50%) PC biosynthesis. Therefore ethanol-dependent decreases in liver cell viability *in vitro* only occur when cells are exposed to high concentrations (100 mmol/L or more) of ethanol for 3 to 4 days. This conclusion is consistent with the observation that cells incubated with 100 mmol/L ethanol for 72 hr have significant alterations in AST release, PC biosynthesis and MTT reduction (Table 1). The alcohol-dependent decrease in cell viability is primarily a result of alcohol metabolism, rather than alcohol *per se*, because 4MP blocks the effects of ethanol (Table 1) on cell viability. This observation (role of metabolism) is important because alcohol dehydrogenase activity rapidly declines in cultured hepatocytes when the medium is not supplemented with hormones (Fig. 2). As a result, alcohol-induced liver cell injury will only be observed in hormone-supplemented cells. It is not apparent whether ethanol is metabolized by both alcohol dehydrogenase (24) and the microsomal ethanol-oxidizing system (24) under these experimental conditions because 4MP inhibits both enzymes (25). However, both systems are probably maintained in these cells because cytochrome P-450– and alcohol dehydrogenase–dependent metabolism of various agents is maintained in cultured hepatocytes under these incubation conditions (26).

Agent-dependent changes in the capacity of hepatocyte monolayers to incorporate labeled glycerol into triacylglycerol are shown in Figure 3. Monolayers were incubated (24 to 72 hr) with 100 mmol/L ethanol in the presence and absence of 0.5 mmol/L oleate (18:1) and 4MP. Oleate or ethanol increases (24 to 48 hr) glycerol incorporation into triacylglycerol; however, the combination of ethanol and oleate increases triacylglycerol formation more than each agent alone. As a result, monolayers incubated (24 to 48 hr) with ethanol, oleate or both readily accumulate fat. However, after 72 hr of 100 mmol/L ethanol exposure, a significant reduction in liver cell function occurs because glycerol incorporation is markedly depressed. All of the ethanol-induced alterations in liver cell triacylglycerol biosynthesis are primarily a result of factors generated during the metabolism of ethanol because they are blocked by 4MP.

Incubation of hepatocyte monolayers for 48 hr with 100 mmol/L ethanol, 0.5 mmol/L palmitate, 0.5 mmol/L linoleate, 0.025 mmol/L VEP or combinations of these agents results in significant alterations in lipid peroxidation (Table 2) and cell viability (Table 1). Ethanol and linoleate alone or in combination increase lipid peroxidation (Table 2) and decrease cell viability (Table 1). However, the combination of ethanol and linoleate potentiates lipid peroxidation (Table 2) and markedly depresses cell viability (Table 1). Palmitate alone or in combination with ethanol decreases lipid peroxidation (Table 2). Palmitate also decreases the ethanol-dependent reduction in cell viability (Table 1). These results suggest that an inverse relationship exists between lipid peroxidation (Table 2) and cell viability (Table 1). This conclusion is supported by the observation that age-dependent increases in lipid peroxidation (Table 3) are associated with marked decreases in liver cell function (PC biosynthesis). Increases in lipid peroxidation that are cell age dependent (Table 3),

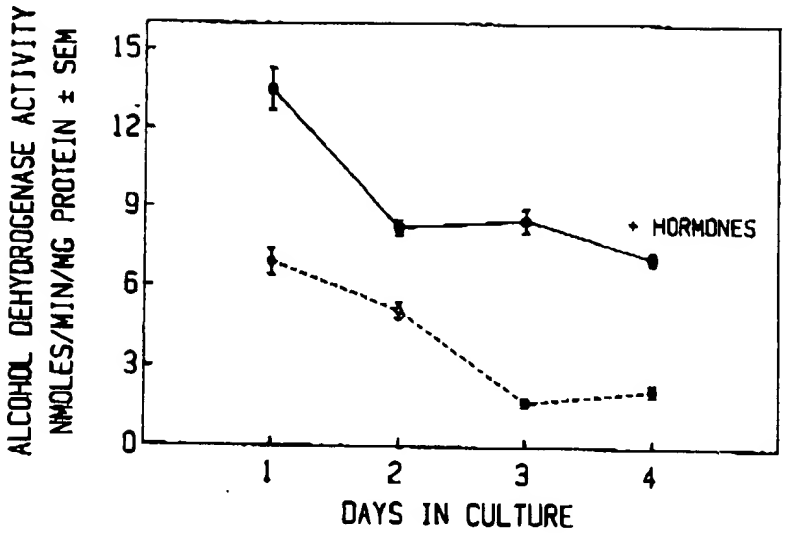

FIG. 2. Alterations in alcohol dehydrogenase activity of hepatocyte monolayers incubated (1 to 4 days) with Waymouth 752/1 medium that is (with hormones) or is not supplemented with estradiol benzoate, testosterone benzoate, dexamethasone and VEP. Each point represents the mean ± S.E.M. of duplicate determinations in three separate monolayers.

TABLE 1. Effect of incubating (72 hr) monolayers with 100 mmol/L ethanol, 0.5 mmol/L linoleate, 0.5 mmol/L palmitate, 0.5 mmol/L 4MP, 0.025 mmol/L VEP or combinations of these agents on cell function

Additions	MTT	AST	[^{3}H]choline PC
Control	107 ± 9[a]	112 ± 14	106 ± 7
100 mmol/L ethanol	51 ± 3[b]	163 ± 21[b]	48 ± 5[b]
0.5 mmol/L 4MP	106 ± 7	114 ± 9	115 ± 11
Ethanol + 4MP	89 ± 9	115 ± 11	98 ± 8
0.5 mmol/L palmitate	103 ± 3	106 ± 5	106 ± 7
Ethanol + palmitate	84 ± 6	132 ± 8	85 ± 8
0.5 mmol/L linoleate	85 ± 8	128 ± 15	89 ± 7
Ethanol + linoleate	11 ± 1[b]	525 ± 35[b]	12 ± 1[b]
0.025 mmol/L VEP	105 ± 6	112 ± 7	155 ± 12[b]
VEP + ethanol	105 ± 6	111 ± 8	115 ± 11
VEP + ethanol + linoleate	85 ± 8	131 ± 18	95 ± 9

[a]All data are based on the percentage of control and are expressed as mean ± S.E.M.
[b]Significance from control is $p \leq 0.01$.

linoleate dependent, ethanol dependent or all three (Table 2); and decreases in cell viability and function (Table 1) are blocked by VEP (antioxidant). Therefore decreases in cellular glutathione (antioxidant) content that are linoleate dependent, ethanol dependent or both (Table 4) might contribute in part to the increases in lipid peroxidation induced by linoleate, ethanol or both (Table 2) because a good correlation ($r = -0.95$) exists between these parameters. In summary, the ethanol-dependent increases in lipid peroxidation (Table 2) may be responsible in part for cell dysfunction because antioxidants (VEP) and saturated (palmitate) fatty acids reduce and unsaturated (linoleate) fatty acids potentiate ethanol-induced cell injury (Table 1).

Hepatocyte monolayers incubated (6 to 72 hr) with 100 mmol/L ethanol display significant changes in PLC activity and PCT activity (Table 5). At 6 and 24 hr of ethanol exposure PCT and PLC activity are significantly increased; however, at 72 hr PCT but not PLC activity is significantly depressed. The ethanol-induced changes in PCT activity (Table 5) correlate well ($r = 0.98$) with the alterations in PC biosynthesis (Fig. 1). All of these ethanol-induced changes in cell functions are primarily a result of factors generated during ethanol metabolism because they are reduced by 4MP (Fig. 3, Table 1).

Hepatocyte monolayers incubated 72 hr with 150 mmol/L ethanol, 0.5 mmol/L linoleate, 0.5 mmol/L 4MP or combinations of these agents display significant changes in PC content (Table 6). Ethanol alone depresses PC content about 30%; however, the combination of ethanol and linoleate markedly reduces (65%) PC content. The ethanol-dependent reduction in PC content is a result of factors generated during ethanol metabolism because 4MP blocks the changes in PC content (Table 6).

VEP prevents the age-dependent (Table 3) and ethanol-induced decreases (Table 1) in liver cell viability. The cytoprotection of VEP may be caused in part by the

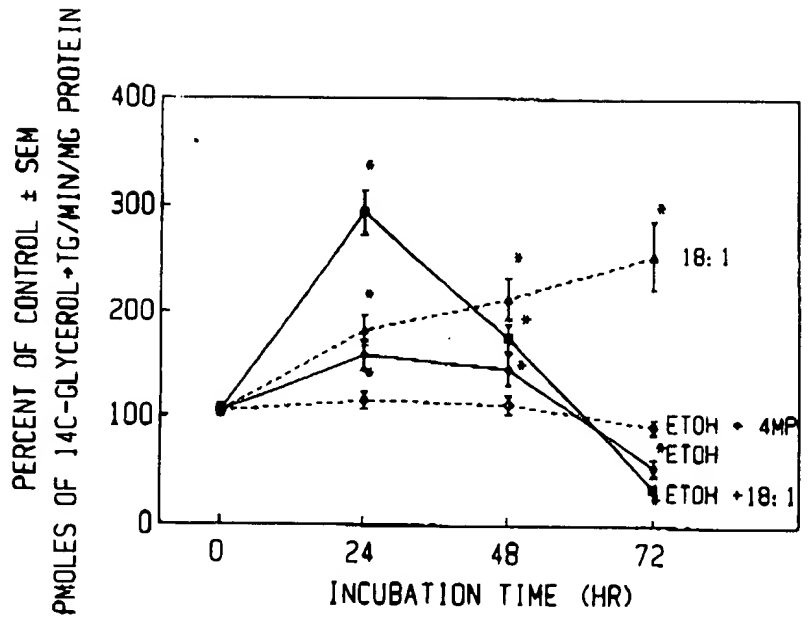

FIG. 3. Changes in the incorporation of [^{14}C]glycerol into triacylglycerol *(TG)* of hepatocyte monolayers incubated (0 to 72 hr) with 100 mmol/L ethanol *(ETOH)*, 0.5 mmol/L linoleate *(18:2)*, 0.5 mmol/L 4MP or combinations of these agents. Each point represents the mean ± S.E.M. of 3 to 9 experimental values. Control values are 154 pmol of [^{14}C]glycerol incorporated into triacylglycerol per minute per milligram protein ± 8. *Significantly different from control ($p \leq 0.01$).

TABLE 2. Alterations in lipid peroxidation of monolayers incubated (48 hr) with 100 mmol/L ethanol, 0.5 mmol/L palmitate, 0.5 mmol/L linoleate or combinations of these agents, in the presence or absence of 0.025 mmol/L VEP

Additions	Without VEP	With VEP
Control	100 ± 4[a]	102 ± 7
100 mmol/L ethanol	146 ± 4[b]	104 ± 2
0.5 mmol/L palmitate	79 ± 4	73 ± 4
0.5 mmol/L linoleate	213 ± 5[b]	131 ± 4
Ethanol + palmitate	126 ± 3	75 ± 5
Ethanol + linoleate	422 ± 21[b]	131 ± 7

[a]All data are based on the percentage of control and are expressed as mean ± S.E.M.

[b]Significance from control is $p \leq 0.01$.

ability of VEP to inhibit lipid peroxidation (antioxidant) induced by either cell age (Table 3), polyunsaturated fatty acids, ethanol or all three (Table 2); and to increase the cell's ability to repair injured membranes by stimulating PC biosynthesis (10) (Table 1) or some combination of these events. Therefore VEP is routinely added (0.010 mmol/L) to the incubation media to reduce the oxyradical-induced reductions in cell function that readily occur in cultured hepatocytes (Table 3). VEP enables the cells to maintain normal cell functions for extended periods (Table 3). Higher concentrations (0.025 mmol/L or more) of VEP will completely prevent the adverse effects of cell age (Table 3), polyunsaturated fatty acids, alcohol or all three on cell function (Table 1).

DISCUSSION

One of the most limiting factors in studies of ethanol-dependent liver cell injury is the absence of an *in vitro* system that mimics the liver cell's response to ethanol *in vivo*, such as fat accumulation and necrosis. This study suggests that primary cultures of adult rat hepatocytes incubated with ethanol may be an appropriate *in vitro* model of ethanol-induced liver cell injury. This conclusion is a result of the observations (Figs. 1 through 3 and Tables 1 through 6) that hepatocyte monolayers incubated (6 to 96 hr) with alcohol display alterations in cell function and viability that are potentiated by unsaturated fatty acids (linoleate) and reduced by 4MP, saturated fatty acids (palmitate) and VEP (Tables 1 through 3). These results suggest that cultured hepatocytes incubated with ethanol can be used to determine the mechanisms by which ethanol induces liver cell dysfunction. Cultured hepatocytes respond appropriately to alcohol exposure *in vitro* because the cells are isolated from phenobarbital-induced rats and maintained in Waymouth 752/1 medium that is supplemented with several hormones and VEP. Under these experimental conditions cultured hepatocytes maintain (4 days or longer), rather than lose, their ability to metabolize various agents such as ethanol, allyl alcohol, cocaine and carbon tetrachloride (20) (Fig. 2). One possible explanation of these observations is that phenobarbital is a broad-spectrum inducer (27) *in vivo*, and the induced cellular processes are more readily maintained *in vitro* by the hormone-supplemented Waymouth 752/1 medium.

The effects of short- and long-term ingestion of

TABLE 3. Alterations in lipid peroxidation and PC biosynthesis of monolayers incubated (0 to 3 days) with or without 0.025 mmol/L VEP

Days	Peroxidation (nmol malondialdehyde/mg protein)		PC biosynthesis (pmol/min/mg protein)	
	Without VEP	With VEP	Without VEP	With VEP
0	0.31 ± 0.07[a]	0.26 ± 0.06	191 ± 6	195 ± 7
1	2.43 ± 0.41[b]	0.25 ± 0.05	64 ± 2[b]	161 ± 11
2	2.13 ± 0.07[b]	0.12 ± 0.01	95 ± 3[b]	174 ± 12
3	2.61 ± 0.10[b]	0.21 ± 0.03	43 ± 1[b]	183 ± 9

[a]All data are expressed as mean ± S.E.M.
[b]Significance from control is $p \leq 0.01$.

TABLE 4. Alterations in glutathione content of monolayers incubated (24 and 48 hr) with ethanol, linoleate and combinations of these agents

Additions	24 hr[a]	48 hr[a]
Control	100 ± 2[b]	100 ± 1
100 mmol/L ethanol	93 ± 1[c]	79 ± 3[c]
0.5 mmol/L linoleate	76 ± 1[c]	67 ± 2[c]
Ethanol + linoleate	78 ± 1[c]	45 ± 1[c]

[a]24- and 48-hr control values are 21.5 and 31.2 nmol glutathione/mg protein, respectively.
[b]All data are based on the percentage of control and are expressed as mean ± S.E.M.
[c]Significance from control is $p \leq 0.01$.

TABLE 5. The influence of incubating (6 to 72 hr) monolayers with 100 mmol/L ethanol on PLC and PCT activity

Incubation time (hr)	PLC	PCT
6	151 ± 5[a, b]	141 ± 8[b]
24	213 ± 15[b]	203 ± 23[b]
48	305 ± 35[b]	74 ± 10
72	287 ± 35[b]	52 ± 4[b]

[a]All data are based on the percentage of control and are expressed as mean ± S.E.M.
[b]Significance from control is $p \leq 0.01$.

ethanol on hepatic triacylglycerol and PC content and biosynthesis are well documented (28-31) in the intact animal. In general, most investigators (28-31) have observed that ethanol intake increases hepatic triacylglycerol and PC content and biosynthesis. These results are consistent with the early effects (24 hr or less) of 100 mmol/L ethanol exposure on hepatocyte monolayer triacylglycerol and PC biosynthesis (Figs. 1 and 3). However, other investigators have noted a decrease in hepatic PC content when long-term administration of ethanol has resulted in liver cell necrosis *in vivo* (32). This ethanol-dependent decrease in PC content and biosynthesis is also observed in monolayers (Fig. 1 and Tables 5 and 6) incubated for 72 hr with ethanol (100 to 150 mmol/L). The differences in hepatic PC content and biosynthesis may represent different phases of ethanol-induced liver cell injury. For example, a rise in PC content and biosynthesis may be a result of a reversible phase (24 hr or less) (Fig. 1 and Table 5), whereas a decrease represents an irreversible phase (72 hr or more) (Fig. 1 and Table 5) of ethanol-induced liver cell injury. This concept might explain why short-term ethanol intake does not result in significant liver damage (reversible injury), whereas long-term ethanol intake is frequently associated with severe liver injury (irreversible injury).

Hepatic PC content is altered by changes in PC biosynthesis, hydrolysis or some combination of these processes. Results shown in Table 5 suggest that PLC activity is rapidly and significantly increased in cells incubated (6 to 72 hr) with 100 mmol/L ethanol. An ethanol-dependent rise in PLC activity might explain

TABLE 6. Alterations in PC content of monolayers incubated (72 hr) with 150 mmol/L ethanol, 0.5 mmol/L linoleate or combinations of these agents in the presence or absence of 0.5 mmol/L 4MP

Addition	Without 4MP	With 4MP
Control	102 ± 6[a]	104 ± 7
150 mmol/L ethanol	73 ± 4[b]	116 ± 11
0.5 mmol/L linoleate	60 ± 15	75 ± 12
Ethanol + linoleate	35 ± 3[b]	80 ± 16

[a]All data are based on the percentage of control and are expressed as mean ± S.E.M.
[b]Significance from control is $p \leq 0.01$.

the changes in hepatocyte monolayer PC biosynthesis and content associated with ethanol exposure in these studies. This conclusion is supported by the observation that cultured hepatocytes incubated with exogenous PLC display exposure time–dependent alterations in PC biosynthesis that are similar to the effects of ethanol on these parameters (33). Hepatic PC biosynthesis is primarily regulated by PCT (34-36). Therefore PCT activity was determined in hepatocyte monolayers incubated (6 to 72 hr) with 100 mmol/L ethanol (Table 5). At 6 and 24 hr of ethanol incubation PCT activity is significantly increased; however, by 72 hr of ethanol exposure PCT activity is reduced by 48%. Roberti et al. (37) also noted that short-term (12 hr) ethanol ingestion increased rat liver PC biosynthesis and PCT activity. These results suggest that a good correlation exists ($r = 0.98$) *in vitro* and *in vivo* between PC biosynthesis (Fig. 1) and PCT activity (Table 5). Apparently, the liver

cell's membrane repair systems (PC hydrolysis is less than or equal to PC biosynthesis) are usually able to repair (reversible) the membrane damage associated with ethanol ingestion *in vivo*; however, when ethanol intake is high and prolonged (long term) the repair systems may be damaged or inadequate (irreversible) to meet the membrane's needs (PC hydrolysis is greater than PC biosynthesis). This latter condition might explain why PC content is depressed in liver cells (Table 6) and animals (with liver damage) exposed for a long time to ethanol (32). If this interpretation is correct, an agent such as VEP may be a potent cytoprotective agent (Tables 1 and 3) because it significantly increases (twofold to fivefold) liver cell PCT activity and PC biosynthesis (10). The phosphate ester of vitamin E is better (fourfold) than succinate (10) or vitamin E, and this difference might be caused in part by the increased capacity of the phosphate ester to partition in the phospholipid bilayer of membranes (38).

The mechanisms by which ethanol induces liver cell injury are still unresolved (39, 40). However, results from this *in vitro* study suggest the following: (a) agents (oxyradicals, acetaldehyde or both) generated during the metabolism of ethanol are responsible in part for liver cell injury *in vitro* (cytoprotection by 4MP) (Tables 1 and 6 and Fig. 3); (b) the depletion of glutathione (Table 4) and stimulation of lipid peroxidation (Tables 2 and 3) may also be important events (cytoprotection by VEP) (Tables 1 through 3); and (c) changes in the hydrolysis, biosynthesis and content of membrane PC are also important (Fig. 1 and Tables 1, 3, 5 and 6). These effects of ethanol *in vitro* are consistent with ethanol-induced changes in the intact animal. For example, short- and long-term ethanol intake rapidly reduces glutathione content and increases phospholipid peroxidation *in vivo* (41-43). The reduction in glutathione content results in the cell's inability to remove toxicants (44, 45) such as activated oxygen species formed during the metabolism of ethanol (40, 46-50). The correlation between glutathione reduction, activated oxygen species and ethanol-induced liver injury *in vivo* and *in vitro* is supported by the work of Strubelt, Younes and Pentz (51). Peroxidation of membrane PC primarily activates cellular PLC (52) (Table 4) rather than phospholipase A_2 (53, 54). The most reasonable interpretation of the peroxidation-dependent activation of cellular PLC is that the cell is trying to reverse (repair) the membrane injury by hydrolyzing the peroxidized PC (33). This interpretation is supported by the observation that inhibitors of phospholipase A_2 (55-58) and PLC (Lamb, Unpublished observation, 1993) do not reduce chemical-induced liver cell injury *in vitro*. However, the hydrolysis of peroxidized-membrane PC would actually increase membrane injury unless PC biosynthesis was correspondingly increased. Therefore the cell must also activate PCT (Table 5) to stimulate PC biosynthesis (Fig. 1). Several reports (33, 36, 59, 60) have clearly demonstrated that cells respond to phospholipase-mediated hydrolysis of membrane PC by activating PCT. When PC hydrolysis and biosynthesis are correspondingly increased (24 hr or less) (Fig. 1 and Table 5), ethanol-induced membrane injury is reversible; however, when PC hydrolysis is greater than PC biosynthesis (72 hr or more) (Table 5), membrane injury is irreversible and the cell dies (Table 1). Therefore chemical-induced changes in the structure (peroxidation or radical addition), hydrolysis and biosynthesis of membrane phospholipids such as PC may be critical events in ethanol-dependent liver cell injury *in vitro*.

The importance of phospholipid peroxidation in ethanol-dependent liver cell injury *in vivo* is supported by the following observations: (a) long-term ethanol intake reduces hepatic vitamin E content and stimulates lipid peroxidation (61-64); (b) ethanol-induced liver alterations are potentiated by vitamin E deficiency (64) and reduced by vitamin E (65, 66); (c) vitamin A administration increases lipid peroxidation (67) and potentiates ethanol-dependent liver injury (68); and (d) unsaturated fatty acids potentiate, whereas saturated fatty acids reduce, ethanol-induced liver injury (5, 6). These *in vivo* results are consistent with the effects of ethanol on liver cell functions *in vitro* (Tables 1 through 6). Therefore phospholipid peroxidation may be a key event in alcohol-induced liver cell injury *in vitro* and *in vivo*. As a result, unsaturated but not saturated fatty acids of membrane phospholipids may be critical targets of active oxygen species such as the hydroxyl radical and superoxide anion. This might explain why saturated fat reduces and unsaturated fat potentiates ethanol-induced liver cell injury *in vitro* (Table 1) and *in vivo* (5, 6). The formation of phospholipid adducts in the membrane disrupts cell function (injury) and activates the cell's defense systems of phospholipid hydrolysis and biosynthesis (repair). Membrane injury is or is not reversible, depending on whether the membrane repair rate is greater than or equal to the rate of membrane injury. Therefore agents such as VEP are potent cytoprotectants because they partition in the phospholipid bilayer of membranes, reduce lipid peroxidation (membrane injury) and stimulate PC biosynthesis (membrane repair).

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Alterations in Phosphatidylcholine Metabolism of Stretch-Injured Cultured Rat Astrocytes

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Abstract: The primary objective of this study was to determine the influence of stretch-induced cell injury on the metabolism of cellular phosphatidylcholine (PC). Neonatal rat astrocytes were grown to confluency in Silastic-bottomed tissue culture wells in medium that was usually supplemented with 10 μM unlabeled arachidonate. Cell injury was produced by stretching (5–10 mm) the Silastic membrane with a 50-ms pulse of compressed air. Stretch-induced cell injury increased the incorporation of [^{3}H]choline into PC in an incubation time- and stretch magnitude-dependent manner. PC biosynthesis was increased three- to fourfold between 1.5 and 4.5 h after injury and returned to control levels by 24 h postinjury. Stretch-induced cell injury also increased the activity of several enzymes involved in the hydrolysis [phospholipase A_2 (EC 3.1.1.4) and C (PLC; EC 3.1.4.3)] and biosynthesis [phosphocholine cytidylyltransferase (PCT; EC 2.7.7.15)] of PC. Stretch-induced increases in PC biosynthesis and PCT activity correlated well ($r \approx 0.983$) and were significantly reduced by pretreating (1 h) the cells with an iron chelator (deferoxamine) or scavengers of reactive oxygen species such as superoxide dismutase and catalase. The stretch-dependent increase in PC biosynthesis was also reduced by antioxidants (vitamin E, vitamin E succinate, vitamin E phosphate, melatonin, and *n*-acetylcysteine). Arachidonate-enriched cells were more susceptible to stretch-induced injury because lactate dehydrogenase release and PC biosynthesis were significantly less in non–arachidonate-enriched cells. In summary, the data suggest that stretch-induced cell injury is (a) a result of an increase in the cellular level of hydroxyl radicals produced by an iron-catalyzed Haber–Weiss reaction, (b) due in part to the interaction of oxyradicals with the polyunsaturated fatty acids of cellular phospholipids such as PC, and (c) reversible as long as the cell's membrane repair functions (PC hydrolysis and biosynthesis) are sufficient to repair injured membranes. These results suggest that stretch-induced cell injury in vitro may mimic in part experimental traumatic brain injury in vivo because alterations in cellular PC biosynthesis and PLC activity are similar in both models. Therefore, this in vitro model of stretch-induced injury may supplement or be a reasonable alternative to some in vivo models of brain injury for determining the mechanisms by which traumatic cell injury results in cell dysfunction. **Key Words:** Cultured astrocytes—Stretch-induced cell injury—Phosphatidylcholine biosynthesis—Phospholipase activity—Phosphocholine cytidylyltransferase activity—Oxyradical scavengers—Antioxidants.
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A limitation in traumatic brain injury (TBI) research is the absence of a simple, widely used in vitro cell model that mimics the cellular effects of traumatic injury in vivo. We have recently developed a model of traumatic injury in brain-derived cells in culture (Ellis et al., 1995). In this model, cells are cultured in tissue culture wells having a 2-mm-thick flexible Silastic bottom. Cell injury is produced by transiently stretching the Silastic membrane and adherent cells with a 50-ms pulse of compressed air. The magnitude and duration of the stretch are easily altered, and this model produces significant and reproducible cell injury, which has been characterized for pure astrocytes, neuronal plus glial cultures, and pure endothelial cell cultures (Ellis et al., 1995; Tavalin et al., 1995; McKinney et al., 1996).

A primary objective of this study was to determine if astrocytes stretched in vitro mimic the effects of TBI on cell function in vivo. An earlier report (Wei et al., 1982) from this laboratory demonstrated that TBI significantly increased brain phosphatidylcholine (PC) biosynthesis and phospholipase C (PLC) activity. Therefore, the influence of stretch-induced cell injury

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Abbreviations used: LDH, lactate dehydrogenase; PC, phosphatidylcholine; PCT, phosphocholine cytidylyltransferase; PEG-SOD, polyethylene glycol-conjugated superoxide dismutase; PLA_2, phospholipase A_2; PLC, phospholipase C; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase; TBI, traumatic brain injury.

on cellular PLC activity and PC biosynthesis was determined as well as the mechanisms by which these cellular changes in phospholipid metabolism occur. These results suggest that (a) traumatic cell injury in vitro and in vivo produces similar alterations in cellular PC biosynthesis and PLC activity, (b) cell injury may be due in part to the interaction of toxic hydroxyl radicals (Haber–Weiss reaction) with the polyunsaturated fatty acids (PUFAs) of membrane phospholipids such as PC, and (c) membrane damage is reversible as long as the cell's membrane repair systems (PC hydrolysis and biosynthesis) are sufficient to repair the membrane PC damaged by oxyradicals. Therefore, determining how traumatic cell injury produces irreversible membrane damage in vitro in stretched cells may be important in understanding how to prevent or reduce cell dysfunction associated with brain injury in vivo.

MATERIALS AND METHODS

Materials

Superoxide dismutase (SOD), catalase, melatonin, vitamin E, vitamin E succinate, arachidonate, vitamin E phosphate, *N*-acetylcysteine, and deferoxamine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cytidine 5′-diphospho[*methyl*-^{14}C]choline, [^{3}H]choline, and [^{3}H]-arachidonate were obtained from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Dimethyl sulfoxide was secured from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Cell culture

Cultured astrocytes were prepared as previously outlined (Amruthesh et al., 1993; Ellis et al., 1995). Astrocytes were isolated from 1–2-day-old rats, processed, and placed in 75-cm^2 flasks for 10–14 days. When cells were confluent, the cells were trypsinized, removed from the flasks, washed, and plated on collagen-coated 25-mm-diameter Silastic membranes, which form the bottom surface of wells in a six-well tissue culture Flex Plate (Flexcell International, McKeesport, PA, U.S.A.). Cells were used for experiments 4 weeks after removal from the rat. In most cases, except where indicated, cells were incubated with 10 μM arachidonate for 4 days before the experiment to enrich the cell's membranes with arachidonate.

Cell injury

Cultured astrocytes were injured by stretching the flexible Silastic membrane (Ellis et al., 1995). The magnitude and duration of the stretch were controlled by a cell injury controller (model 94A; Commonwealth Biotechnology, Richmond, VA, U.S.A.). A 50-ms pressurized pulse (compressed air) of variable magnitude was used to produce a 5.7-, 6.3-, 7.5-, or 8.6-mm Silastic membrane deformation. This corresponds, respectively, to a 31, 38, 54, or 72% stretch. The Silastic membrane is stretched and returns to the control unstretched position in ~250 ms. Cells were stretched in normal culture medium, which in some cases had various agents added 1 h before the induction of injury. Within 5 min after the cells were injured, labeled choline was added to the medium. Incubations with labeled choline were continued for 90 min at 37°C in a 95/5% air/CO_2 incubator. All additions were made in concentrated stock solutions and were diluted in media to produce the indicated concentrations.

Isotope incorporation

After the appropriate incubation period, a 0.02-ml aliquot of [^{3}H]choline was added to the media to give a final concentration of 0.1 mmol/L (0.1 μCi). Incubations were stopped after 90 min by addition of 2 ml of methanol. Cells were removed from the Silastic membrane by scraping with a Teflon-coated spatula and placed in glass test tubes. Two milliliters of chloroform and 0.5 ml of 0.08 *M* HCl were then added to each test tube. Labeled PC was extracted and washed by a technique modified from that of Bligh and Dyer (1959). Radioactivity was determined using a β-counter (Beckman Bioanalytical Systems Group, Fullerton, CA, U.S.A.). The control rate (in picomoles per minute per milligram of protein) of choline incorporation into PC varied significantly (twofold) between preparations. As a result, most data are expressed as a percentage of the control so that data from several experiments could be combined. Data represent mean ± SEM values of at least six to nine experimental determinations. Statistical significance was determined by Student's *t* test or one-way ANOVA.

Arachidonic acid release

Cultured astrocytes were also incubated 24 h before stretch-induced injury with [^{3}H]arachidonate (0.5 μCi per well). Approximately 92–96% of the labeled arachidonate was incorporated into cellular phospholipids. After the labeling period, cells were washed three times and placed in 1.0 ml of Dulbecco's phosphate-buffered saline supplemented with 1 m*M* glucose and 1.0% fatty acid-free bovine serum albumin. Cells were then injured by stretching (6.3 mm). Release of radiolabeled arachidonate into the incubation medium was measured 0–60 min postinjury. For these experiments, the medium was removed, and lipid was extracted (Bligh and Dyer, 1959). The lipid extracts were chromatographed on silica gel G plates using the solvent system of petroleum ether/diethyl ether/glacial acetic acid (82:18:1 by volume). Neutral lipids, mono-, di-, and triacylglycerols, free fatty acids, and phospholipids were each identified by cochromatography of authentic lipid standards, scraped into scintillation vials, and counted for radioactivity. Results are expressed as a percentage of total cell-incorporated radioactivity released as free fatty acid (Rzigalinski and Rosenthal, 1994).

Enzyme assays

Phospholipase A_2 (PLA_2; EC 3.1.1.4) and PLC (EC 3.1.4.3) were quantified by techniques previously described (Lamb and Schwertz, 1982; Coleman et al., 1988). One hour after injury, cell medium was aspirated, and reactions were initiated by adding 0.5 ml of medium containing 28 m*M* Tris-HCl (pH 8.3), 5.4 m*M* ATP, 1.1 m*M* dithiothreitol, 0.08 m*M* CoA, 50 m*M* NaF, 5.4 m*M* $CaCl_2$, 0.7 m*M* linoleate (in dimethyl sulfoxide), and 0.05 m*M* [^{14}C]-glycerol-3-phosphate (0.1 μCi). Cells were then placed in a 95:5 air/CO_2 incubator at 37°C for 30 min. Reactions were terminated by placing the plates on ice, and cells were scraped from each well with a Teflon-coated spatula. Scraped cells were placed in glass test tubes, and 4 ml of $CHCl_3$/methanol (1:1 vol/vol) containing 1% 1 *M* HCl was added along with 1.3 ml of water. Lipids were extracted and washed by a technique modified from that of Bligh and Dyer (1959) and separated by silicic acid column chromatography as pre-

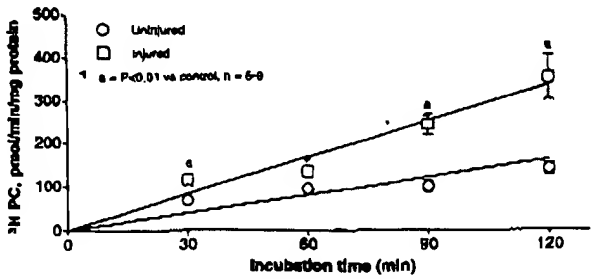

FIG. 1. Influence of incubation time (0–120 min) on incorporation of labeled choline into PC of injured (□; 7.5 mm) and control (○) astrocytes. Unless indicated otherwise, astrocytes were enriched with 10 μM arachidonate for 4 days before the experiments were conducted. Data are mean ± SEM (bars) values, in pmol of PC formed/mg of protein, of five to nine determinations from three different cell preparations. $^{a}p < 0.01$, values significantly different from control values.

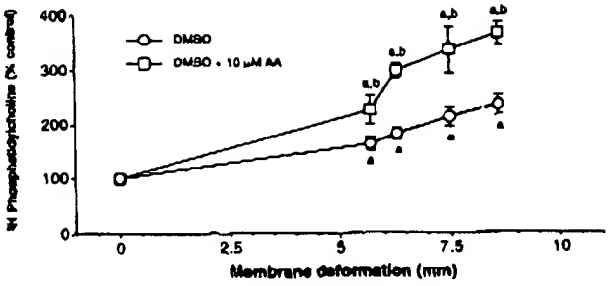

FIG. 3. Influence of the degree of injury and arachidonate (AA) enrichment on PC biosynthesis in cultured astrocytes: cells enriched with AA (□) and cells incubated with vehicle [dimethyl sulfoxide (DMSO)] only (○). Data are mean ± SEM (bars) percentages of control values from nine determinations. $^{a}p < 0.05$, values significantly different from control values; $^{b}p < 0.05$, significant differences between AA-enriched and nonenriched cells.

viously described (Lamb and Fallon, 1970) with slight modifications. Silicic acid columns loaded with labeled lipids were washed three times with 2 ml of $CHCl_3$ first, then $CHCl_3$/methanol (2:1 vol/vol) and then $CHCl_3$/methanol (1:1 vol/vol) to elute diacylglycerol, phosphatidic acid, and lysophosphatidic acid, respectively. The activities of PLC and PLA_2 are expressed as a percentage of the amount of [^{14}C]phosphatidate converted to diacylglycerol and lysophosphatidate per minute per milligram of protein, respectively. Phosphocholine cytidylyltransferase (PCT; EC 2.7.7.15) activity was measured as previously described (Ishidate et al., 1980; Weinhold et al., 1986) with slight modifications. One hour after injury, the medium was aspirated, and 0.5 ml of medium containing 2.4 mM phosphorylcholine, 15 mM magnesium acetate, 60 mM NaCl, 70 mM Tris-HCl (pH 7.5), 1.2 mM EDTA, 0.3 mM PC/oleate suspension, and 0.65 μCi of [^{14}C]phosphorylcholine was added to each plate and incubated for 30 min at 37°C in a 95:5 air/CO_2 incubator. Reactions were terminated by adding 0.2 ml of 10% trichloroacetic acid to each plate. Cells were then scraped off the plate with a Teflon-coated spatula and placed in glass test tubes, and labeled CDP-choline was extracted as previously described (Ishidate et al., 1980; Weinhold et al., 1986). PCT activity is expressed as the nanomoles of CDP-choline formed per minute per milligram of protein.

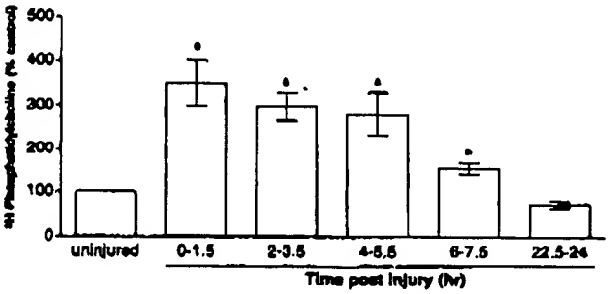

FIG. 2. Influence of time postinjury on astrocyte PC biosynthesis. Data are mean ± SEM (bars) percentages of control values from nine determinations. $^{a}p < 0.01$, values significantly different from control values.

Protein was quantified as outlined previously (Lowry et al., 1951) using bovine serum albumin as a standard.

Lactate dehydrogenase (LDH) enzyme release

Stretch-induced cell injury was measured by the release of intracellular LDH. Cell medium was removed 2 h after injury and analyzed spectrophotometrically using an LDH kit obtained from Sigma. LDH remaining in the cells was also quantified after treating the cells with Triton X-100 (1 ml of 0.2%). The stretch-induced LDH release was expressed as a percentage of the total releasable LDH (Ellis et al., 1995).

RESULTS

Unless indicated otherwise, cells are stretched by a 7.5-mm Silastic membrane deformation produced by a 50-ms pulse of compressed air. Compared with control cells, stretched cells have a two- to threefold increase ($p \leq 0.01$) in the incorporation of [^{3}H]choline into PC throughout a 120-min incubation period (Fig. 1). PC biosynthesis is linear for 120 min in both stretched and control cells (Fig. 1). The rapid and significant stretch-induced increase in cellular PC biosynthesis is maintained for at least 7.5 h postinjury and returns to control levels by 24 h (Fig. 2).

As membrane deformation (5.3–8.6 mm) and cell injury increase, there is a significant ($p \leq 0.01$) rise in cellular PC biosynthesis (Fig. 3). PC biosynthesis is also increased in stretched cells enriched for 4 days with 10 μM arachidonate; however, the magnitude of the stretch-induced increase in PC biosynthesis is about twofold higher in arachidonate-enriched cells (Fig. 3). The release of cellular LDH is also significantly ($p < 0.01$) greater, 18 and 27%, in arachidonate-enriched cells that are stretched 6.3 and 7.5 mm, respectively. These results suggest that arachidonate-enriched cells are more susceptible to stretch-induced injury than nonenriched cells.

Significant (threefold; $p \leq 0.01$) increases in PC biosynthesis produced by stretch injury are completely blocked by an iron chelator (1 mM deferoxamine) and

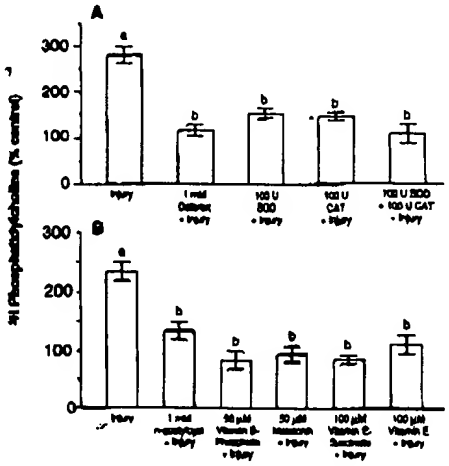

FIG. 4. A: Influence of deferoxamine (Deferox; 1 mM), SOD (100 U), catalase (CAT; 100 U), and a combination of SOD and CAT on the stretch-induced (7.5 mm) increase in cellular PC biosynthesis. B: Influence of antioxidants [1 mM *N*-acetylcysteine (n-acetylcyst), 50 μM vitamin E phosphate, 50 μM melatonin, 100 μM vitamin E succinate, and 100 μM vitamin E] on the stretch-induced increase in cellular PC biosynthesis. All the agents were added to the cell medium 1 h before stretch injury. Data are mean ± SEM (bars) percentages of control of nine determinations. $^{a}p < 0.01$, values significantly different from noninjured cells; $^{b}p < 0.01$, values significantly different from injured cells.

scavengers of reactive oxygen species (ROS) such as catalase (hydrogen peroxide), SOD (superoxide anion), or a combination of both scavengers (Fig. 4A). The stretch-induced increase in PC biosynthesis is also reduced by antioxidants such as *N*-acetylcysteine, vitamin E phosphate, melatonin, vitamin E succinate, and vitamin E (Fig. 4B). Vitamin E phosphate and melatonin were the most potent antioxidants under these experimental conditions (Fig. 4B). These results (Fig. 4) suggest that cell injury may be due in part to the production of hydroxyl radicals by an iron-catalyzed Haber–Weiss reaction (McCord and Day, 1978).

The activity of several enzymes involved in the hydrolysis (PLA_2 and PLC) and biosynthesis (PCT) of membrane phospholipids such as PC is increased in stretched cells (Table 1). All enzyme activities are

TABLE 1. *Stretch-induced alteration in the activity of PLC, PLA_2, and PCT in astrocytes*

Enzyme	Activity (pmol/min/mg of protein)	
	Control	Injured
PLC	0.40 ± 0.02	0.53 ± 0.04[a]
PLA_2	0.83 ± 0.11	1.48 ± 0.21[a]
PCT	1.70 ± 0.40	5.00 ± 0.60[a]

Data are mean ± SEM values of at least 10 determinations.
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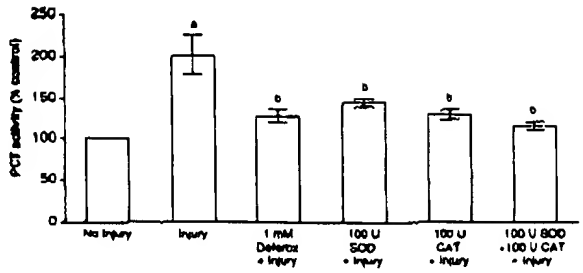

FIG. 5. Effect of 1 mM deferoxamine (Deferox), SOD (100 U), catalase (CAT; 100 U), and a combination of SOD and CAT on the stretch-induced (7.5 mm) increase in cellular PCT activity. All agents were added to the medium 1 h before cells were injured. Data are mean ± SEM (bars) percentages of control values from nine determinations. $^{a}p < 0.01$, values significantly different from noninjured cells; $^{b}p < 0.01$, values significantly different from injured cells.

increased significantly ($p \leq 0.02$); however, PLA_2 and PCT activities are increased more than PLC activity. These stretch-induced increases in enzyme activity may be due in part to the interaction of hydroxyl radicals with the PUFAs of membrane PC. This conclusion is supported by the observation that deferoxamine, SOD, catalase, and a combination of SOD and catalase reduce the stretch-dependent increases in PCT activity (Fig. 5). There is an excellent correlation ($r = 0.983$) between stretch- and agent-dependent alterations in PC biosynthesis (Fig. 4A) and PCT activity (Fig. 5).

By 10 min postinjury, there is a threefold increase in [^{3}H]arachidonate release from the membrane phospholipids of stretched cells compared with control cells (Fig. 6). The release of arachidonate gradually increases in a linear fashion throughout the 60-min incubation period (Fig. 6), and >1% of the total radiolabeled arachidonate incorporated into cellular lipid is released by 15 min. The actual mass of arachidonate released may be somewhat greater because these observations reflect only release of incorporated radiolabeled arachidonate. Nevertheless, these results suggest that cellular phospholipids are rapidly hydrolyzed in

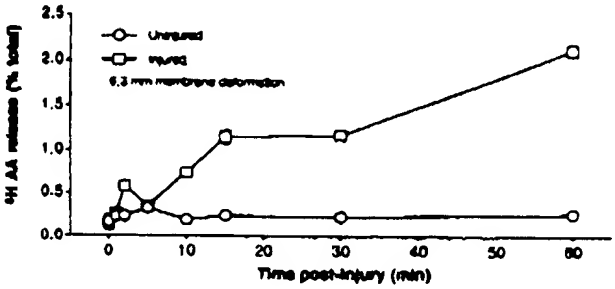

FIG. 6. Release of [^{3}H]arachidonate ([^{3}H]AA) from control (○) and injured (□) cultured astrocytes. Cells were prelabeled with [^{3}H]AA and then injured by stretching (6.3 mm) and compared with noninjured control cells. Data represent the percentage of [illegible]

stretched cells as a result of an increase in PLA_2 activity.

DISCUSSION

The primary objective of this study was to determine if cultured astrocytes stretched in vitro mimic the increases in cellular phospholipase activity and PC biosynthesis produced by TBI in vivo (Wei et al., 1982; Shohami et al., 1989). This study suggests that stretch-induced cell injury rapidly increases astrocyte phospholipase activity and PC biosynthesis. Because both in vitro and in vivo models of traumatic cell injury produce rapid and significant increases in cellular PC biosynthesis and phospholipase activity, cultured astrocytes stretched in vitro appear to be a relevant model for determining the mechanisms by which TBI produces alterations in cell PC hydrolysis and biosynthesis.

Various studies suggest that cell injury associated with TBI is due in part to ROS such as superoxide anion and hydroxyl radical (Kontos and Wei, 1986; Hall et al., 1993; Siesjö, 1993). This conclusion is supported by the observations that (a) free radical scavengers such as SOD (Muizelaar et al., 1993) and lipid antioxidants (Hall et al., 1991; Hall, 1993) reduce brain dysfunction associated with TBI and (b) TBI increases the production of superoxide anion (Kontos and Wei, 1986) and hydroxyl radical (Hall et al., 1993; Sen et al., 1994). These observations suggest that ROS are involved in cell injury; however, there is little agreement about the source of ROS and their mechanism of cell injury.

PUFAs of cellular membrane phospholipids are abundant and readily accessible targets of oxyradicals. Oxyradicals can injure membranes by initiating the peroxidation of PUFAs or adding directly to an unsaturated chemical bond of the PUFA (Reynolds and Molsen, 1980). In both cases, the alteration of membrane phospholipids results in a disruption of membrane functions such as enzyme activity, chemical transport, and signal transduction. To survive, the cell must rapidly repair the damaged membrane by stimulating the sequential hydrolysis and biosynthesis of radicalized membrane phospholipids such as PC. The cell contains cytosolic enzymes (PLC, PLA_2, and PCT) that can rapidly translocate to membranes as needed to activate PC hydrolysis and biosynthesis (Lamb et al., 1988). This might explain why TBI is associated with a rapid increase in PLC activity (Wei et al., 1982), PLA_2 activity (Shohami et al., 1989), and PC biosynthesis (Wei et al., 1982). The rapid and significant increase in the cellular level of the toxic hydroxyl radical by TBI (Hall et al., 1993) might alter membrane phospholipids and activate cellular phospholipases because radicalized PUFAs are readily hydrolyzed (Gamache et al., 1988; Huband and Lamb, 1995). These in vivo effects of TBI on cellular phospholipid metabolism are similar to those observed in stretch-injured astrocytes (Figs. 1–5) because deferoxamine, SOD, and catalase reduce the stretch-induced increase in PC biosynthesis (Fig. 4A) and PCT activity (Fig. 5). These results suggest that traumatic cell injury in vitro and in vivo may be due in part to formation of hydroxyl radicals by an iron-catalyzed Haber–Weiss reaction (Haber and Weiss, 1934; McCord and Day, 1978). This conclusion is supported by the observation that antioxidants and SOD reduce cell dysfunction associated with traumatic cell injury in vitro (Fig. 4) and in vivo (Hall, et al., 1991; Hall, 1993; Muizelaar et al., 1993).

The subcellular source of the oxyradicals produced by TBI is unresolved. Potential sources of oxyradicals include mitochondria and arachidonate metabolism. Stretched cells rapidly release arachidonate (Fig. 6) from membrane phospholipids, and superoxide anion would be produced if the released arachidonate was metabolized by cellular lipoxygenase and cyclooxygenase (Ellis et al., 1981; Kukreja et al., 1986; White and Krause, 1993). Stretch-injured cells also display significant alterations in mitochondrial structure ranging from swelling to complete disruption (Ellis et al., 1995). It is known that mitochondria are capable of producing ROS (Younes, 1988). Therefore, cellular ROS levels could be increased by several mechanisms; however, the rate and magnitude of ROS production suggest that mitochondrial disruption would be the most likely source of ROS. As a result, it is proposed (Fig. 7) that stretching cells disrupts mitochondria and releases ROS into the intracellular compartment. Superoxide anion, hydrogen peroxide, and redox active iron may then interact to produce the toxic hydroxyl radical by an iron-catalyzed Haber-Weiss reaction (Haber and Weiss, 1934; McCord and Day, 1978). The hydroxyl radical would interact with the PUFAs of membrane PC and disrupt membrane function. Membrane repair occurs when cytosolic phospholipases translocate to injured membranes and hydrolyze the radicalized PC (Lamb et al., 1988, 1994), and cytosolic PCT translocates to the PC-depleted membranes and stimulates PC biosynthesis (Lamb et al., 1988, 1994; Sanghera and Vance, 1990; Yao et al., 1990). Therefore, oxyradical-induced membrane damage is or is not reversible, depending on whether the membrane repair rate (PC metabolism) is greater than or equal to the rate of membrane injury.

The role of arachidonate in stretch-induced cell injury is unresolved. Cells enriched with arachidonate are more susceptible to stretch-mediated alterations in LDH release and PC biosynthesis (Fig. 3). One explanation of these results is that membranes enriched with arachidonate are more susceptible to oxidative injury (Spector and Yorek, 1985; Wey et al., 1993) because they have more unsaturated sites for oxyradical attack. Alternatively, more arachidonate may be available as substrate for lipoxygenase and cyclooxygenase enzymes. The metabolism of arachidonate might potentiate the oxidative stress associated with TBI by increas-

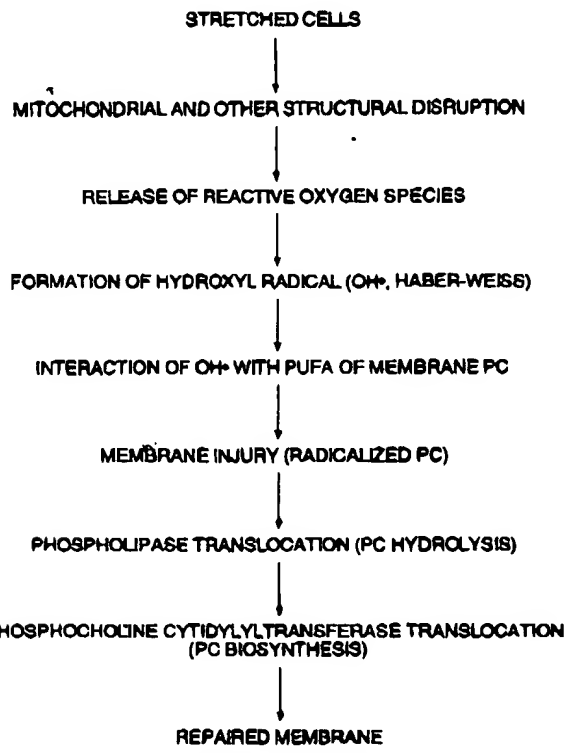

FIG. 7. Proposed "oxyradical theory of stretch-induced membrane injury." Stretching cells disrupts the structure of various organelles such as mitochondria, which results in the release of ROS (superoxide and hydrogen peroxide) into the cytosol. The toxic hydroxyl radical is formed by an iron-catalyzed Haber-Weiss reaction ($H_2O_2 + O_2^{\cdot -} \xrightarrow{Fe} OH^{\cdot} + OH^- + O_2$). Hydroxyl radicals interact with the PUFAs of membrane phospholipids such as PC and may initiate lipid peroxidation by abstracting a hydrogen, binding directly to the unsaturated chemical bonds (radical addition), and forming PC adducts or some combination of both peroxidation and radical addition. Membrane injury (radicalized PC) is reversible if cytosolic phospholipases and PCT translocate to the site of membrane injury and stimulate PC hydrolysis and biosynthesis, respectively.

ing the production of ROS such as superoxide (Chan et al., 1988).

This laboratory has recently examined (McKinney et al., 1996) the effect of polyethylene glycol-conjugated SOD (PEG-SOD) on uptake of propidium iodide by stretch-injured cultured astrocytes, neuronal plus glial cultures, and endothelial cell cultures. In this earlier study, PEG-SOD did not reduce the cellular uptake of propidium iodide, a measure of stretch-induced cell injury, in astrocytes or neuronal plus glial cells but did reduce propidium iodide uptake by stretch-injured endothelial cells. These previous results contradict the current astrocyte findings; however, there is a reasonable explanation. Propidium iodide is a relatively insensitive marker of cell injury because extensive membrane damage must occur before this dye enters the cell. Therefore, it is unlikely that free radical scavengers such as PEG-SOD could alter the extensive damage to membranes produced by stretching that results in dye uptake and nuclear staining of astrocytes unless the magnitude of free radical generation was very high and caused extensive damage. We believe this is the case in endothelial cells because they are known to produce high levels of oxyradicals and their uptake of propidium iodide is reduced 50% by PEG-SOD (McKinney et al., 1996). In the current study, biochemical markers (phospholipases and cytidylyltransferases) that are more sensitive than propidium iodide uptake were used to assess stretch-induced cell injury. Our results suggest that oxyradicals produced by stretch-injured cells alter these biochemical parameters. Thus, scavenging oxyradicals reduces free radical interactions with astrocyte membrane phospholipids and subsequent activation of phospholipase and PC biosynthesis but does not prevent the more gross damage to astrocyte membranes measured by propidium iodide uptake.

In summary, these studies suggest that traumatic cell injury may be due in part to the interaction of hydroxyl radicals with the PUFAs of membrane phospholipids such as PC. Membrane injury is reversible as long as the rate of membrane injury is less than the membrane's repair rate (PC metabolism). Therefore, any agent that can reduce the rate of membrane injury, increase the membrane's repair rate, or both is a potential cytoprotective agent. In this regard, agents that gain access to injured cells to chelate redox active iron or scavenge free radicals (SOD, antioxidants, etc.) should, in a theoretical sense, be beneficial in the treatment of TBI (Muizelaar et al., 1993; White and Krause, 1993).

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